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The transcriptional regulation of the Colony-Stimulating Factor 1 Receptor (*csf1r*) gene during hematopoiesis

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1. ABSTRACT

The colony-stimulating-factor 1 receptor (CSF-1 R) is a tyrosine kinase receptor that is absolutely required for macrophage differentiation and thus occupies a central role in hematopoiesis. Mice deficient for the *csf1r* gene show multiple defects in macrophage development, reproduction and tissue remodeling. Moreover, deregulation of this gene is a hallmark of many tumors. This includes repression of expression in acute myeloid leukemia and aberrant activation in certain solid tumors, such as breast cancer. Expression of this gene therefore needs to be tightly controlled. This review summarizes experiments providing a detailed picture of how transcription of *csf1r* gene expression is regulated. Aside from the direct relevance to hematopoiesis, studies of *csf1r* transcriptional regulation provide a model for understanding the molecular mechanisms that control mammalian cell fate.

2. OVERVIEW

The coordinate and regulated expression of cell type- and cell stage-specific genetic programs requires the establishment of an active chromatin structure within specific genes at the correct differentiation stages, as well as the heritable inactivation of genes expressed in alternative cell fates. The hematopoietic system has been extensively studied as a model for understanding mammalian cell fate decisions. All types of mature blood cells originate from hematopoietic stem cells (HSCs) which have the potential to self-renew or progress into the various differentiation pathways specific for different blood cell lineages. HSCs express a lineage promiscuous gene expression program which is restricted once committed precursor cell types are formed. Blood cell lineage specification is controlled by the balance of specific transcription factors in hematopoietic precursor cells (27,54). These transcription factors interact with genes organized in specific chromatin architectures, and the

assembly of transcription factor complexes on cis-regulatory elements initiates chromatin remodeling and modification events. The cooperative action of these diverse protein assemblies leads to the stable establishment of differential genetic programs (reviewed in (4)). A major focus of research in recent years has been to unravel the molecular basis of cell lineage specification at the epigenetic level. One important reason for this is that it is now recognized that tumor formation commonly involves epigenetic reprogramming of the normal cell lineage from which the cancer cell is derived and the deregulation of tissue specific gene expression programs ont a large scale.

Studies of the transcriptional regulation of cell lineage-restricted genes have provided much of the insight into the molecular mechanisms by which cell lineage specification occurs. The expression of such genes tends to reflect how the entire network of transcription factors and signaling molecules behaves in response to developmental cues and other outside signals. A clear example is the extensive literature on the transcriptional control of the α – and β-globin loci. These genes are not crucial for the formation of the erythroid lineage, but analyses of their regulation identified transcription factors, such as GATA1 and EKLF, that are required for erythropoiesis as a whole (73). In the macrophage lineage, the gene encoding the receptor for colony-stimulating-factor 1 (*csf1r)* has been studied as a model. Expression of *csf1r* is absolutely required for this developmental pathway, and this is reflected in the fact that it is regulated by a set of transcription factors that by themselves are crucial for myeloid development. In this review we will summarize our recent advances regarding the regulation of *csf1r* and what these experiments teach us about general principles of cell fate decisions in the hematopoietic system.

3. GROWTH AND DIFFERENTIATION IN THE MONONUCLEAR PHAGOCYTE SYSTEM ARE CONTROLLED BY COLONY-STIMULATING-FACTOR 1 AND ITS RECEPTOR

The macrophage populations of many organs are seeded during embryonic development (49), and there is ongoing interest in whether cells in particular organs, such as lung and brain, proliferate locally or are continuously replenished from the blood. With that proviso, most tissue macrophages turn over, and are replaced by blood monocytes, which in turn are derived from a committed progenitor shared with granulocytes, the common myeloid progenitor. These cells ultimately arise from HSCs, which in the adult mammal reside in the bone marrow.

Tissue macrophages may represent 10-15% of the total cells in many organs of the body. Their appearance, gene expression profile and function is very heterogeneous, and the family of cells includes microglia in the brain, antigen-presenting dendritic cells associated with most epithelia and mucosal surfaces, and bone-resorbing osteoclasts (34,35). Owing to their extensive functional differences, there are few gene products that are common to all members of the MPS. In fact, many surface markers, such as the integrins CD11b and CD11c, lectin-like molecules such as sialoadhesin and macrosialin, the G protein coupled receptor, EMR1 (F4/80), and certain chemokine receptors e.g. CCR1, CCR2 and CX3CL1) are used rather arbitrarily to divide the MPS into putative functional subsets (25). One molecule that is expressed on the vast majority of cells designated as mononuclear phagocytes is the receptor for macrophage colony-stimulating factor, CSF-1, which is a type III integral member protein tyrosine kinase encoded by the c-fms proto-oncogene (*csf1r*). The ligand, CSF-1 controls the proliferation, differentiation, adaptation and survival of cells of the mononuclear phagocyte system (12,77). In mice, a natural mutation of the *csf1* gene, the osteopetrotic mutation (op/op), or an introduced knockout of the *csf1r* gene, causes a very substantial reduction in mononuclear phagocyte numbers in most tissues of the body. In addition, the op/op mice are osteopetrotic because of the lack of bone-resorbing osteoclasts. Osteoclast numbers increase with age in these mice, in part due to partial compensation by other growth factors, *vegfa* or *flt3l* (52). Even though the op/op and *csf1r* KO mice are viable, the importance of CSF-1-dependent macrophages in development is indicated by a failure to thrive, and deficiencies in development of the central nervous system, pancreas, mammary gland and male and female reproductive function (12). The majority of the phenotypic defects seen in the *op/op* mice including reproductive defects and perturbations in organ development, are even more penetrant in *csf1r* knockout mice (13), possibly reflecting the availability of maternally-derived CSF-1 in the case of the op/op. A similar phenotype is associated with mutation of the *csf1* gene in the tl/tl rat (15). These experiments demonstrate that the CSF-1 pathway is a central part of the transcription factor and signaling network regulating macrophage development.

4. REGULATION OF CSF-1 RECEPTOR SURFACE AND mRNA EXPRESSION

Aside from macrophages, the other definitive site of *csf1r* expression is the placental trophoblast. CSF-1 is produced in large amounts during pregnancy, but its actions on trophoblast cells do not appear to be absolutely required for trophoblast development. Instead, CSF-1 seems to act on trophoblasts to elicit a protective immune response against transplacental infections, as exemplified by studies on the response to listeria monocytogenes (29). In this respect, trophoblasts, although of completely distinct developmental origin, share with macrophages a function in innate immunity. In addition to trophoblasts, many studies have detected expression of *csf1r* mRNA or CSF-1R protein in human tumors, especially breast, ovarian and endometrial tumors, and such expression has been correlated with poor prognosis and progression (1,2,3,5,8,9,10,11,17,18,26,37,39,40,41,42,43,44,45,46,51, 55,60, 61,62,63,66,71,75,82, 83,84,85,88). This expression could reflect roles of *csf1r* in normal development in the female reproductive system. It is not clear whether CSF-1 signaling per se contributes to tumorigenesis or progression; at least in a subset of cases the receptor is co-expressed with the ligand, CSF-1.

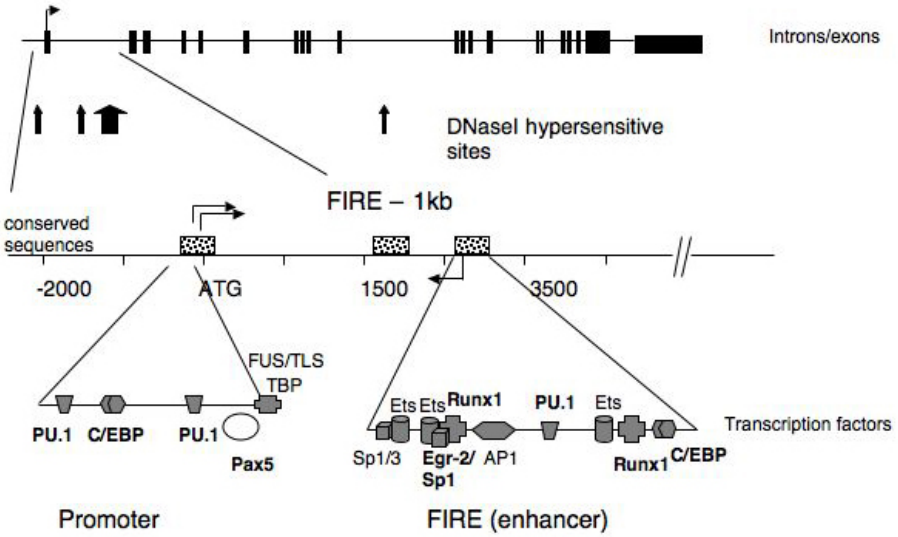

Figure 1. Genomic structure of the mouse *csf1r* locus with introns and exons (top diagram). Conserved DNA sequences, chromatin structure and transcription factor binding sites are indicated below. DNase I hypersensitive sites (DHSs) are indicated as large black arrows, the mRNA transcription start site is depicted as a small black arrow in top diagram and the antisense RNA transcription start site initiating at FIRE is shown as small arrow pointing in the opposite direction. Transcription factors that have been shown to bind *in vivo* are indicated in bold.

Within the hematopoietic system, CSF-1R surface expression is one of the earliest events in myeloid lineage commitment. Receptor protein expression is only found on committed macrophage precursors (CFU-Ms). However, CSF-1R mRNA expression does not provide a definitive macrophage marker. Low levels of *csf1r* mRNA expression are detected already in HSCs and expression persists in the entire multipotent progenitor compartment (78,81). This includes committed lymphoid progenitor cells and common myeloid progenitor cells (CMPs). mRNA expression is switched off in all non-macrophage cell types with the exception of granulocytes which express the mRNA, but do not produce CSF-1R protein. Surprisingly, granulocytes can produce the protein when cultured in vitro, and will then respond to CSF-1 by differentiating into macrophages (64). In addition, there appears to be extensive post-transcriptional regulation. The FANTOM mouse transcriptome project revealed that there are multiple 3' end truncations and internal splice variants of the mRNA that may encode secreted and membrane-anchored, kinase-dead forms of the protein (22,6,7).

Csf1 mRNA expression is induced in a wide range of infectious, inflammatory and malignant pathologies (12,77), and directly controls the expression of numerous downstream effectors in macrophages. Macrophages recruited in response to sterile inflammatory stimuli may actually be autocrine for CSF-1 (38). Accordingly, many macrophage regulators act in part by modulating CSF-1 action through intersection with the CSF-1R. For example, there is substantial family of genes that is repressed by CSF-1 and induced as a direct consequence of the removal of CSF-1 signaling in response to lipopolysaccharide (68,69,76). Lipopolysaccharide, the protein kinase C agonist PMA, and CSF-1 itself all down-regulate transcription of *csf1r* mRNA (89).

5. STRUCTURE OF THE *CSF1R* LOCUS, CIS-REGULATORY ELEMENTS AND TRANSCRIPTION FACTORS DRIVING *csf1r* EXPRESSION

The mammalian *csf1r* gene is a member of a family of genes encoding type III protein tyrosine kinases, along with the gene encoding the platelet-derived-growth-factor receptors A and B (PDGFRA and B) and the receptor for stem cell factor, c-kit. All four have similar intron-exon structures, and appear side-by-side as pairs (*PDGFR-B* and *csf1r*, *PDGFR-A* and *c-kit*), suggestive of an ancestral duplication and reduplication. The *csf1r* gene is within a substantial region that is syntenic across most mammals. As the number of available completed mammalian genome sequences expands, examination of the ECR browser (http://ecrbrowser.dcode.org/) highlights the overall conserved architecture, and hot spots of high sequence similarity within non-coding regions amongst mammalian species (discussed below). Interestingly, between the *PDGFR-B* and *csf1r* loci, there is a processed ribosomal protein L7 pseudogene that is highly conserved across all mammalian species.

The full complement of regulatory elements required for correct regulation of the *csf1r* locus was defined by three approaches: the identification of sequences conserved between species to identify potential regulatory elements, the mapping of DNaseI hypersensitive chromatin sites (DHSs) to identify functional elements, and the analysis of transgenic mice harboring different combinations of these elements. Figure 1 shows a summary of the position of conserved sequences and DHS and also the position of binding sites for different transcription factors which have been identified to date. The specific features and function of each element are summarized below.

5.1. The *csf1r* promoter

In murine macrophage cells, the *csf1r* promoter displays three closely spaced DHSs (33) whereas the human promoter only shows one (19). This is probably due to the fact that the mouse promoter has two microsatellite inserts which separate three clusters of conserved regions. The alignment across species reveals some sequence conservation extending around 400bp upstream of the major start site cluster, after which no alignments can be discerned between mouse and human for at least 5kb (http://ecrbrowser.dcode.org/). The *csf1r* macrophage promoter has no TATA box, and initiates transcription at multiple sites, thereby falling within the so-called broad class of transcription start sites defined in the genome-wide classification based upon CAGE tags (6). The individual transcription start sites within the start site cluster conform in general to the pyridimine-purine minimal initiator consensus (6). However, unlike the large majority of broad class promoters, but in common with many other myeloid-expressed genes, the *csf1r* promoter is not GC-rich and there is no CpG island in the vicinity.

One of the key questions that arise with respect to the function of this class of promoter is how the basal transcription machinery is assembled in the absence of a TATA box, or of GC-rich sequences that can substitute for this element. Immediately adjacent to the dominant start site cluster in all species is a loose repeat of CAG or CAA triplets. We have purified macrophage nuclear proteins that bind specifically this sequence in both mouse and human, and identified the related Ewing sarcoma and FUS/TLS protein (Hume, DA et al., submitted). Both proteins are TATA-associated factors, and share an RNA recognition motif and zinc finger domain. One previous report demonstrated that FUS/TLS binds DNA through the zinc finger, and shares binding site specificity with the so-called myeloid zinc finger protein (56). We hypothesize that the two proteins substitute for TATA-binding protein in recognizing the start site region (although TBP is still part of the transcription initiation complex, and can be identified in ChIP (47).

Immediately upstream of the EWS/FUS/TLS site is a set of purine-rich sequences that contain multiple binding sites for the macrophage-specific Ets family transcription factor, PU.1. In the mouse and rat, the promoter contains a GT repeat, upstream of which is a further PU.1 site which is actually the strongest binding site in the mouse (59), but which is absent in most other species. A multimerised PU.1 recognition site is able to function as a minimal macrophage-specific promoter, but activity requires cooperation between PU.1 and another Ets family member (59). There are at least 15 other members of the Ets family expressed in mouse macrophages and at least 6 others can either trans-activate, or repress the activity of the mouse promoter (unpublished). The precise architecture of the PU.1 sites across species is quite divergent, and it is not clear which subsets bind PU.1 and which bind other Ets factors. This uncertainty is reflected in the controversy around the precise phenotype of PU.1 knock-out mice. While in one particular knock-out mouse PU.1 is absolutely required for macrophage differentiation and expression of *csf1r* mRNA during development (14, 66) this is not the case for another independently derived mouse line that shows some degree of macrophage differentiation and *csf1r* expression (49). This may be explained by the finding that there is evidence of a variable penetrance of the PU.1 knockout phenotype in different genetic backgrounds (50).

Upstream of the purine-rich block, there is conserved block which is annotated in human as the RUNX1/CEBP site. This pair of motifs was shown to be essential for human *Csf1r* promoter activity in transfections, and to bind the RUNX1 and CEBP-alpha (90). Interestingly, the mouse and rat genes have sequence variants in both motifs that abolish binding of both factors. The mouse promoter is sensitive to the related C/EBP transcription factor, C/EBPß (89), which binds to an adjacent site found only in the rodent *csf1r* promoters. Interestingly, the mouse has a perfect Runx1 recognition site upstream of the GT repeat, adjacent to the PU.1 site, which could indicate that the function has been retained in a distinct location. Chromatin immunoprecipitation experiments have indeed demonstrated binding of Runx1 to the *csf1r* promoter in myeloid precursor cells (C.Bonifer and H.Krysinska, unpublished observation).

The maximal activity of the mouse *csf1r* promoter in transfections of macrophage cell lines requires only the 300bp upstream of the start codon, or 200bp distal to the most prominent start site. However, this conclusion has the limitation that available transfectable cell lines in mouse (RAW264) or human (THP-1) express *csf1r* mRNA at much lower levels than primary macrophages and are not, themselves, CSF-1 dependent. Upstream of the 300bp promoter, the next 200bp are conserved to a lesser extent across species, but there is a clear alignment (64). This region contains all of the trophoblast-specific transcription start sites that have been identified in the mouse gene, but this provides no clear explanation for the sequence conservation, since human trophoblast do not utilize this region for transcription. Promoter constructs containing the 500bp region are active in a wide range of mouse tumor cells, and are stimulated by CSF-1 signaling when the receptor is expressed on a fibroblast background. Two AP1 sites could contribute to this activity. Hence, the upstream region contains growth factor-responsive activity that could be involved in early macrophage differentiation as well as trophoblast expression. In an attempt to elucidate the importance of this region, we have deleted 150bp from -300 to -450 in the context of a 7.2kb *csf1r*-EGFP transgene (see below). Data from multiple lines suggest that the region is absolutely required for trophoblast expression, but may also be needed for maximal macrophage expression (unpublished observation).

5.2. The Fms intronic regulatory element (FIRE) and creation of a *csf1r* transgene

The 300bp mouse *csf1r* promoter alone had significant macrophage-restricted promoter activity, but longer promoter constructs had significant activity in a wide range of tumor cell lines, and this was correlated with the production of *csf1r* transcripts that extended into the first intron. Further analysis of conserved regions and also

chromatin studies led to the identification of other key regulatory elements in the first intron of the *csf1r* gene, notably a 300bp segment that is very highly-conserved across mammalian species. The activity of this element in both the mouse and the human *csf1r* genes is marked by a very strong DHS in macrophages (33,20). This sequence, which we call the Fms intronic regulatory element (FIRE), has macrophage-specific enhancer activity in transient transfections. The central importance of the intron, and FIRE in particular, was demonstrated by the production of a series of transgenic mouse lines in which the promoter alone, the promoter plus first intron, or the intron-containing construct with FIRE removed, were used to direct expression of an EGFP reporter gene (64). The 7.2fms promoter containing the intron has been used to direct myeloid-specific expression of a number of different transgenes, and has been remarkably position and copy number independent in those applications. Within the intron, there is a second, somewhat less conserved, enhancer element that has not been examined in detail (33,64). There has also not been any systematic mutagenesis of FIRE, although the alignments across species provide a strong indication of the likely functional elements. However, we could show that one of the Sp1 sites which can also bind Egr-2 is essential for enhancer activity of FIRE (47) (see below). Interestingly, Egr-2, but not Sp1 is present in precursor cells, whereas Sp1 dominates in mature cells (unpublished observation).

Aside from enhancer activity, FIRE has reverse promoter activity that is comparable to the forward activity of the major macrophage promoter. An antisense transcript starting at FIRE that is indicative of promoter activity is detected in macrophages. Interestingly, in mouse B cells antisense transcription can be detected in the absence of mRNA synthesis (80). In mouse macrophages, reverse promoter activity is induced by stimuli such as LPS, phorbol esters or CSF-1 that act to inhibit *csf1r* transcription (manuscript in preparation). Antisense transcription is also detected in human macrophages (unpublished), but the actual transcribed region of the intron upstream of FIRE is not conserved at all across species. These observations suggest that the antisense transcript, or antisense transcription per se, may be involved in down-regulating sense mRNA transcription. However, its precise function has not yet been elucidated.

6. THE DEVELOPMENTAL REGULATION OF *CSF1R* EXPRESSION

6.1 *csf1r* activation during macrophage differentiation

We performed a series of experiments that investigated the order of events taking place during the developmental activation of the *csf1r* locus. As outlined above, *csf1r* mRNA expression can be detected in HSCs but expression is low and levels do not differ from those of CMPs (78,80). Studies of transcription factor occupancy by DMS *in vivo* footprinting at *csf1r* cis-regulatory elements in HCSs and CMPs demonstrated that in both cell types the promoter was fully occupied. This was not the case for FIRE. When CMPs were differentiated into macrophages *in vitro* the chromatin status of FIRE changed with time (78). CSF-1R surface protein expression was only detected in committed macrophage precursor cells when transcription factor assembly at FIRE was complete.

These assays were highly informative but the small numbers of primary cells limited the kinds of analysis that can be performed. This problem was circumvented using a mouse line derived from the fetal liver of PU.1 -/- mice which did not express *csf1r* mRNA and which carried an inducible form of the PU.1 protein (47, 87). Induction of PU.1 in these cells restored macrophage differentiation and permitted detection of the precise order of events occurring during the activation of *csf1r* from the silent state (47). In this differentiation system *csf1r* expression was also activated in two steps. mRNA levels were only detectable after about 24 hours and significant CSF1-R surface expression required two days of in vitro differentiation. In contrast, as shown by chromatin immunoprecipitation (ChIp) analyses and *in vivo* footprinting, transcription factor assembly and chromatin remodeling at the promoter was complete after 6 hours, and this included binding of PU.1, Runx1, and C/EBP. *Csf1r* did not contain acetylated histones. The only mark of active chromatin was a low level of histone H3 lysine 4 methylation that indicated ongoing or recent transcription. As in primary cells, factor assembly and chromatin remodeling at FIRE was only complete after about 48 hours. FIRE activation was paralleled by the recruitment of the histone acetyl-transferase CBP with a concomitant increase in histone acetylation across the whole locus and recruitment of the SWI/SNF component brg1. Interestingly, the delay in assembly was observed with all transcription factors that bound to the promoter and FIRE, including PU.1 itself. One explanation for this biphasic activation is that PU.1 is necessary to induce the expression of secondary transcription factors (48) including the Egr-2 and JunB (74). The Egr-2 site is essential for FIRE activity and overlaps with an Sp1 site. By ChIP analysis, Egr-2 bound to FIRE, but only after PU.1 induction. The same was also true for c-Fos which is a potential partner for JunB and could bind to a functional AP1 site within FIRE (unpublished results). These results provide an explanation for restricted expression of CSF-1 receptor in committed macrophage precursor cells. Although the *csf1r* promoter is primed in HSCs, FIRE is not yet fully active and mRNA expression levels are low. As outlined in Figure 2, high levels of *csf1r* mRNA, the acquisition of active chromatin marks and full chromatin remodeling are only seen after all transcription factors are present that are required for full FIRE activity. This two-step activation mechanism ensures that although *csf1r* is already expressed in HSCs, high levels of *csf1r* mRNA and CSF-1 receptor protein are only expressed in cells destined to be responsive to CSF-1 signaling.

6.2 Silencing of *csf1r* in lymphoid lineages and the role of Pax5

During B lymphopoiesis *csf1r* mRNA expression is switched off. B cell development proceeds via committed lymphoid progenitors (CLPs), which still can give rise to all lymphoid cell types, followed by committed B cell precursors such as pro-B cells. Figure 2 shows the different

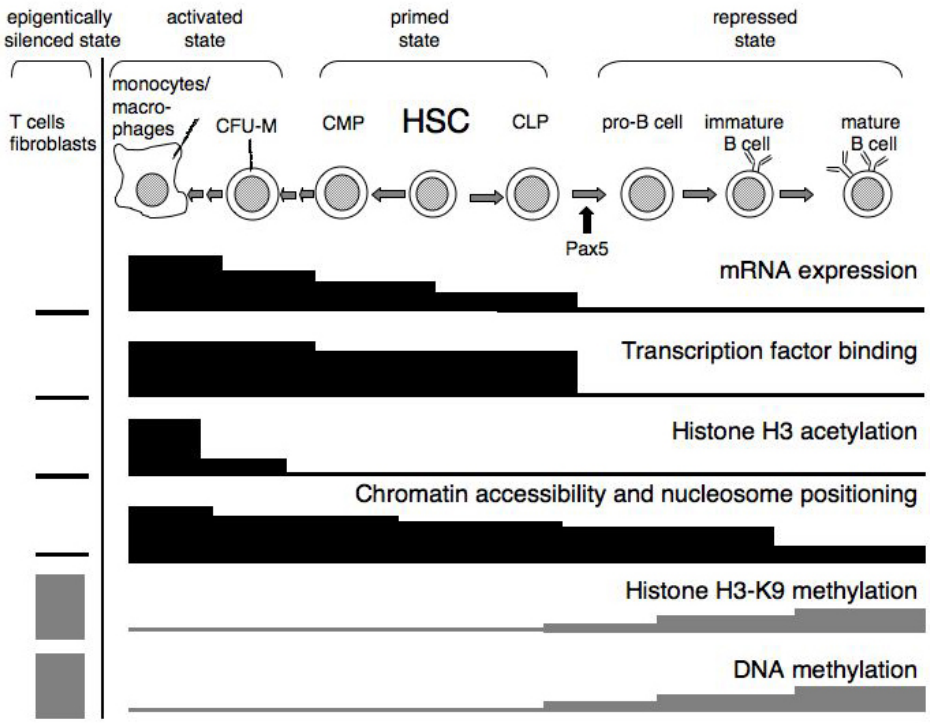

Figure 2. Alteration of different chromatin features during the Pax5 mediated silencing of *csf1r* during B lymphopoiesis as compared to *csf1r* mRNA expressing cells (macrophages) and cells where *csf1r* is truly epigenetically silenced (fibroblasts). CSF-1R surface protein expression is indicated on committed macrophage precursor cells (CFU-M) and macrophages.

stages of *csf1r* silencing at the epigenetic level. CLPs still express *csf1r* mRNA and the gene is still occupied by transcription factors (80). After the CLP stage transcription factor binding and DNaseI hypersensitivity are lost and mRNA expression from the *csf1r* promoter ceases. Although these processes are completed at the pro-B cell stage, *csf1r* chromatin is still in a partially active conformation and accessible to DNaseI. The promoter nucleosome, which is remodeled in cells expressing *csf1r,* is still in the partially active conformation, i.e. the transcription start sites are exposed, whereas in T cells and fibroblasts the nucleosome covers the actual mRNA transcription start sites. Another interesting observation is that DNA methylation - which is low in HSCs - is increased in T cells, but stays low in B cells. Here, *c-fms* promoter and enhancer elements remain unmethylated throughout. Ongoing antisense transcription from FIRE is reflected in elevated levels of H3K4-tri-methylation throughout the intronic regulatory region. In summary, these experiments demonstrate that silencing of *csf1r* in the lymphoid lineage occurs via different mechanisms in B cells and T cells. While *csf1r* is truly epigenetically silenced in T cells and fibroblasts, it is in a partly active chromatin conformation in B cells. The reason for this turned out to be that B cells express PU.1 and *csf1r* in the B cell lineage needs to be actively repressed by the B cell specific transcription factor Pax5. Pax5 is required for the maintenance of B cell identity, meaning that it is crucial for the activation of a B cell specific gene expression program as well as for the repression of lineage inappropriate genes (53). In the absence of Pax5, B cell development is blocked at the pro-B cell stage, and these cells express a lineage promiscuous gene expression program, including *csf1r*. In conditional Pax5 knock-out mice *csf1r* was re-expressed even in mature B cells if Pax5 was deleted (80). Pax5 represses *csf1r* by binding directly to a specific DNA sequence at the *csf1r* promoter overlapping with the main transcriptional start sites (79); the same site is recognized by the EWS and Fus/TLS factors discussed above. Binding of Pax5 leads to an immediate loss of RNA polymerase binding to the *csf1r* promoter. The DNA binding domain of Pax5 is sufficient for repression, indicating that it does not need to recruit co-factors but instead interferes with binding of the basal transcription machinery by steric hindrance. Using a cell line carrying an inducible Pax5 protein in a Pax5 null background demonstrated that induction of Pax5 led to an immediate removal of RNA polymerase II followed by the loss of upstream transcription factors. This occurred without major changes in the histone modification pattern, confirming the results with primary B cells. Interestingly, the same experiments demonstrated that Pax5 also targeted FIRE, but transfection experiments demonstrated that FIRE does not add to *csf1r* repression by Pax5. We were also unable to demonstrate direct binding of Pax5 to FIRE (unpublished). The role of Pax5-FIRE interaction is therefore currently elusive.

Taken together, our data demonstrate that *csf1r* is primed in HSCs, but that this priming involves mainly the promoter. In B cells, this residual expression is eliminated by Pax5 which interferes with Pu.1 transactivation and

actively represses the *csf1r* promoter. This dynamic interaction between activators and repressors is probably the reason why *csf1r* chromatin is not epigenetically silenced and *csf1r* is easily reactivated after the conditional inactivation of Pax5. Interestingly, in both myeloid and lymphoid precursor cell types which are represented by PU.1-/- and Pax5-/- cells, *csf1r* chromatin appears to carry neither positive nor negative modifications and DNA at *csf1r* cis-elements is unmethylated. From our data it appears as if *csf1r* chromatin is in a "neutral" modification state, and only becomes extensively modified once FIRE mediates high-level transcription, where we find a high level of histone acetylation or when it is epigenetically silenced in the absence of activators and histones carry the H3 lysine 9 methyl mark (81).

7. *CSF1R* AND CELL CYCLE REGULATION

Regulation of *csf1r* expression is coupled to the cell cycle. The minimal promoter of *csf1r* in mice is responsive to growth factor signaling, including signaling from the CSF-1R itself, in transient transfections (16). Another finding demonstrating cell cycle coupling came from an unexpected angle. Retinoblastoma (Rb) knock-out mice have defects in fetal erythropoiesis. Interestingly, deletion of the bHLH protein Id2 rescues this phenotype, and it was subsequently shown that a failure in terminal macrophage differentiation contributes to this phenotype as macrophages are involved in the maturation of erythroid cells. Macrophages interact with developing erythroblasts via a receptor protein (Emp) and this interaction is required for erythroblast maturation and nuclear extrusion (72,36). Iavarone et al., (36) showed that Id2 directly blocks PU.1 activity, but also interacts with Rb. In the absence of Rb, PU.1 is sequestered by Id2 and cannot bind to its cellular targets, indicating that the three proteins are in balance. One of these targets is *csf1r*, and due to this functional growth factor receptor knockout, macrophage precursors are formed, but mature macrophages able to support erythropoiesis cannot develop. The binding of Id2 to Rb, and of PU.1 to Rb, is controlled by the phosphorylation state of Rb, which in turn in regulated by cyclins, and is thus coupled to mitogenic signaling and cell cycle progression. These intriguing results provide a direct link between cell cycle regulators and the expression of *csf1r*, suggesting an intricately balanced feedback loop controlling cell growth and differentiation. Interestingly, using conditional Rb knock-out mice, it was also shown that these mice display a myelodysplasia, which is an extensive proliferation of myeloid precursors in the absence of differentiation, further strengthening the possible link between lack of Rb and terminal macrophage differentiation (86).

A secondary link between expression of CSF-1R and the cell cycle is evident from studies on CSF-1-dependent bone marrow-derived macrophages (BMM). CSF1-R promoter activity in transfections is activated by the transcription factor Ets2 (59), which is inducible by CSF-1R signaling (23). Runx1 (AML1) mRNA, which acts upon both the promoter and FIRE, was found to be repressed by CSF-1 signaling, and induced upon growth factor removal, in parallel with levels of *csf1r* mRNA (31). CSF-1R signaling leads to activation of jun kinase phosphorylation, and BMM were found to require jun kinase activity absolutely for survival. This in turn was linked to a relationship between jun kinase activity and PU.1 protein stability (32).

9. *CSF1R* AND LEUKEMIA

As outlined above the CSF-1 receptor protein has been implicated in tumorigenesis and in itself can act as an oncogene (70,16). The oncogenic potential of CSF-1R in leukemia was originally demonstrated in a murine model of myelo-monoblastic leukemia (24) although at present there is little convincing evidence linking overexpression of the gene and human leukemia. However, recently the first translocation involving *CSF1R* was described that fused the RNA Binding Motif 6 (RBM6) gene to the CSF-1R gene and this fusion protein is capable of inducing a myeloproliferative disease (28). In addition, in recent years it has become apparent that deregulation of the human *CSF1R* gene may be an important phenotype contributing to leukemogenesis. In addition, *CSF1R* is a target of leukemic RUNX1 fusion proteins such as RUNX1-ETO and has served as an important target gene to unravel the mechanism of action of this oncoproteins.

Reduced or absent expression of *CSF1R* with a concomitant disruption of macrophage differentiation accompanies certain types of acute myeloid leukemias (AML). The causal link has been supported by mouse models. For example, the reduction of expression of PU.1 causes an AML in mice which is characterized by the absence of *csf1r* expression (58). We could show that the leukemogenic fusion protein RUNX1-ETO that is produced by the t(8;21) translocation acts as a repressor of *CSF1R* expression by binding to FIRE (19). Patients with a t(8;21) translocation express *CSF1R* at a level that is identical to that observed in wild-type CD34 precursor cells and have little or no monocytic differentiation (21). Interestingly, although leukemic blasts cells are blocked at an early stage of myeloid differentiation, *in vivo* footprinting experiments demonstrated that *CSF1R* cis-regulatory elements including FIRE are occupied by transcription factors. Moreover, FIRE displays a chromatin signature that is normally only seen in mature myeloid cells such as a strong DNaseI hypersensitive site (20,21). This indicates that RUNX1-ETO represses *CSF1R* and probably also a number of other genes in the context of an open chromatin structure, which is plastic and supports gene expression This proposition was elegantly proven by experiments that demonstrated that inactivation of RUNX1-ETO by RNAi or peptides designed to disrupt RUNX1-ETO co-repressor interactions led to upregulation of *CSF1R* expression and macrophage differentiation (30, 57).

10. PERSPECTIVE

Although we now have a very clear picture of the transcriptional regulation of *csf1r*, many questions are still open. For example, we know very little about the molecular details of how CSF-1 signaling feeds back on its *csf1r*

expression and which role this feedback plays in macrophage differentiation. This involves the elucidation of all signaling molecules and transcription factors responding to CSF-1 dependent signaling pathways. We do not know the molecular mechanism whereby cell cycle regulators impact on *csf1r* chromatin and whether cytokine signaling, CSF-1R expression and macrophage differentiation are linked. The latter question is particularly important for our understanding of the role of *csf1r* expression in leukemia.

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