MECHANISMS IN ENDOCRINOLOGY

Pioneer transcription factors in pituitary development and tumorigenesis

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Abstract

Pioneer transcription factors have key roles in development as master regulators of cell fate specification. Only a small fraction of all transcription factors have the pioneer ability that confers access to target genomic DNA sites embedded in so-called 'closed' heterochromatin. This ability to seek and bind target sites within the silenced portion of the epigenome is the basis for their role in changing cell fate. Upon binding heterochromatin sites, pioneer factors trigger remodeling of chromatin from a repressed into an active organization. This action is typically exerted at enhancer regulatory sequences, thus allowing activation of new gene subsets. During pituitary development, the only pioneer with a well-documented role is Pax7 that specifies the intermediate lobe melanotrope cell fate. In this review, a particular focus is placed on this Pax7 function but its properties are also considered within the general context of pioneer factor action. Given their potent activity to reprogram gene expression, it is not surprising that many pioneers are associated with tumor development. Overexpression or chromosomal translocations leading to the production of chimeric pioneers have been implicated in different cancers. We review here the current knowledge on the mechanism of pioneer factor action.

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The essence of pioneer action

Pioneer factors are transcription factors that have, in addition to the usual properties of other transcription factors (TF), the unique ability to recognize their target DNA sequence within condensed, so-called 'closed', chromatin or heterochromatin, and to trigger opening

of this chromatin (1, 2). This unique property allows pioneer factors to implement new programs of gene expression through opening of the chromatin landscape at regulatory elements, such as enhancers and promoters. Consequently, this property enables pioneer factors to act

Invited Author's profile

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as master regulators of cell differentiation and cell fate; hence, they are key regulators in development. Of the thousands of TFs, about two handfuls are currently known to have pioneer activity with a spectrum of properties that suggest there may well be different types of pioneers. Already, two types of pioneers can be distinguished; first, the so-called pluripotency factors (eg Sox2, Oct4) that trigger large-scale chromatin rearrangements and second, the pioneers involved in cell differentiation that primarily target enhancers for chromatin opening. A common property of both types is their ability to recognize target DNA sequences in heterochromatin (heterochromatin is the highly compacted portion of the (epi)genome that is typically inactive and with its DNA heavily methylated

at CpG dinucleotides), but it is yet to be determined whether the nature of the permissive heterochromatin for each type of pioneer is the same. After recognition of DNA targets within heterochromatin, the time course of chromatin opening by pioneers is relatively slow (Fig. 1). In complex cases such as the reprogramming of induced pluripotent cells (iPS), this occurs over weeks in tissue culture models. Whereas the nature of the chromatin changes associated with chromatin opening, namely the switch from a repressed chromatin organization toward an active chromatin, is described to some extent for many pioneers, the underlying mechanisms remain largely unknown. This review first discusses the properties of various pioneer factors with a particular emphasis on one

Figure 1

Mechanism of pioneer transcription factor action. The mechanism of pioneer transcription factor action is illustrated using data derived from analyses of Pax7 action in pituitary AtT-20 cells. In this corticotrope model cell line, Pax7 action leads to cell fate trans-differentiation into a melanotrope-like fate (4). The unique aspect of pioneer action resides in recognition of target DNA sequences within heterochromatin (defined below) that is typically marked by DNA methylation and the histone mark H3K9me2 (6). This is followed by stabilization of Pax7 binding and deposition of the active enhancer mark H3K4me1. Chromatin opening is completed by appearance of DNA accessibility and deposition of the active enhancer mark H3K27ac. For the Pax7-dependent melanotrope set of enhancers, this is associated with recruitment of other nonpioneer transcription factors such as Tpit. Target gene expression follows. The time course provided on the left that were determined using an inducible system in AtT-20 cells. Finally, epigenetic memory is established through demethylation of enhancer DNA. Heterochromatin is the highly compacted or closed chromatin portion of the (epi) genome that contains inactive or repressed genes in a given cell type.

pioneer extensively studied in pituitary development, Pax7. The implication of various TFs in early pituitary development is discussed in the context of their putative pioneer actions and finally, the implications of pioneer factors in tumorigenesis is reviewed.

Recognition of pioneered genomic DNA target sites is dependent on DNA sequence recognition constraints that are similar to the binding of the same TFs to sites within open chromatin. Throughout this review, we refer to 'pioneered' sites as those sites that are opened following pioneer-dependent chromatin remodeling. Indeed, the analysis of DNA sequence motifs recognized by pioneers at their pioneered targets did not show significant differences of motif conservation compared to their transcriptional targets (3, 4). This appears to be true for all pioneers studied so far but an interesting case is the pioneer Pax7. This pioneer has two DNA binding domains (DBD), one paired domain and one homeodomain. Each Pax7 DBD can recognize a cognate DNA sequence with most target enhancers containing either motif or both. The characterization of enhancers pioneered by Pax7 in pituitary cells revealed a composite motif that contains a paired motif juxtaposed to a homeodomain motif: this composite motif appears to be a higher affinity site and the frequency of its occurrence at pioneered enhancers is enriched compared to other enhancers (4, 5). This suggests that higher affinity DNA sites may favor the pioneering process; however, these composite sites are not present at all pioneered enhancers and the DNA sequence requirements for pioneering therefore appear more complex. Altogether, the conserved DNA sequence motifs found at pioneered enhancers suggest that pioneer:DNA interactions in themselves are not different or a critical factor in order to allow pioneer action.

The discriminating feature of pioneers allowing them to recognize their targets in heterochromatin may therefore be their ability to interact with critical heterochromatin proteins as discussed in the next section. This ability may account for the observation that many pioneers have large subsets of genomic binding sites (far more than regular nonpioneer TFs as observed in ChIPseq, Fig. 2A) where binding is not associated with any chromatin change (3, 6, 7). These sites thus appear to be resistant to the pioneering action (6). For FoxA, this subset of binding sites is associated with DNA binding motifs that are less conserved than the level of conservation observed at transcriptional targets (3). It was proposed that interactions at these degenerate sites may represent a scanning mechanism to scout throughout heterochromatin for appropriate pioneer sites. Sequence

motif degeneration was however not observed for Pax7 at similar subsets of sites. These differences may be related to intrinsic differences in the mechanisms of heterochromatin interactions for different pioneers.

Pioneer interactions with nucleosomes and chromatin

Nucleosomes are the basic units of chromatin condensation and they carry the post-translational modifications (PMT) that define euchromatin and heterochromatin. Chromatin opening by pioneer factors involves the replacement of repressive PMT marking heterochromatin by marks associated with active enhancers. Repressed or closed heterochromatin is typically marked by di- or tri-methylation of lysine 9 of histone H3 (8). These H3K9me2 and H3K9me3 marks are historically associated with facultative and constitutive heterochromatin, respectively, and both types of heterochromatin are packed into regular arrays of nucleosomes. In contrast, active chromatin is depleted of these repressive marks and instead, marked with the activating PTMs H3K4me1 at enhancers and H3K4me3 at promoters (9). Notably, H3K9me3-enriched heterochromatin domains seem to constitute a barrier to pioneer binding as they impair Oct4 and Sox2 recruitment, except if proteins involved in maintenance of H3K9me3 are knocked-down (10). These domains are associated with higher-order chromatin condensation and are crucial for proper developmental lineage specification (11).

At active regulatory elements, activating marks are further associated with depletion of nucleosomes that is typically revealed by techniques that measure DNA accessibility. These techniques include DNase sensitivity, FAIREseq and mostly used today, the technique of ATACseq (assay for transposase-accessible chromatin, Fig. 2B) (12). Transcriptionally active enhancers are also marked by the presence of the general coactivator p300 that has histone acetylase activity responsible for the deposition of the H3K27ac mark at active enhancers (9). Thus, pioneer factors initiate the process of switching chromatin organization from repressive to active state, leading to enhancer activation and expression of novel subsets of genes. Enzymes and remodeling protein complexes responsible for implementing many of these marks were characterized and are reviewed elsewhere (13, 14). Also, multiple pioneer factors were shown to recruit and/or be dependent on the recruitment of ATP-dependent chromatin remodelers such as the SWI/SNF

Figure 2

The pioneer action. The assessment of pioneer action is achieved using primarily two genome-wide techniques followed by high throughput DNA sequencing. (A) The ChIPseq technique uses an antibody to assess the presence of epitopes on chromatin; these epitopes may be transcription factors or histone marks that define different chromatin states. (B) The technique of ATACseq (Assay for Transposase-Accessible Chromatin using sequencing) relies on the ability of the transposase Tn5 to cleave and tag double-stranded DNA when chromatin structure allows DNA access. Fragments liberated through the transposase's action are sequenced to map genomic regions that exhibit DNA accessibility. (C) ChIPseq and ATACseq profiles are shown for the landmark enhancer of the *PCSK2* gene that is opened and activated following Pax7 pioneering. The ChIPseq for Pax7 shows its binding to the enhancer sequence: this is associated with appearance of an ATACseq peak, deposition of the active enhancer mark H3K4me1, recruitment of the general coactivator p300 and finally recruitment of the transcription factor Tpit. Tpit does not itself exhibit pioneer activity and requires prior action of Pax7 for its recruitment to the enhancer. The lesser Tpit peak co-migrates with Pax7 whereas the stronger peak reveals the position of a palindromic DNA binding Tpit site (4).

complex for chromatin opening (15, 16, 17). The exact temporal sequence of chromatin changes occurring during pioneer factor action remains however largely undefined in contrast to the clear description of the before and after chromatin states (Fig. 2C).

Numerous studies of enhancer chromatin landscapes identified differences between enhancers that have the potential to be active compared to enhancers that are actively involved in transcription (9). The former group may be considered as primed and is marked by H3K4me1 whereas enhancers actively involved in transcription are usually also marked by p300 and H3K27ac. Active enhancers also exhibit nucleosome depletion in their center as revealed by DNA accessibility (eg by ATACseq). Pioneer action is associated with these two subsets of

enhancers: for Pax7, pioneering results in priming of an enhancer subset whereas another subset is fully activated (6). This observation suggests that these two enhancer states define sequential steps in enhancer activation.

Condensed heterochromatin is characterized by DNA wrapped around nucleosomes and further compacted by linker histones H1 (18). In addition to the presence of the repressive histone marks described above, it is also decorated by a group of specific chromatin-associated proteins such as HP1 $\alpha/\beta/\gamma$ (19) or KAP1 (Tif1 β /Trim28, (20)). HP1 α for instance recognizes the repressive H3K9me2/3 mark and is thereby recruited to heterochromatin regions. By homodimerizing, it bridges nearby nucleosomes and promotes chromatin condensation and spread of the condensation (19). HP1 α also has the ability to lead

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to heterochromatin phase separation, thus capturing the chromatin in an environment that would favor its condensation and the recruitment of other repressive factors (21). KAP1 is crucial for heterochromatin formation and maintenance as it mediates the recruitment of repressive histone methyltransferases involved, among others, in the deposition of H3K9me2/3 (20). Altogether, this chromatin organisation is classically viewed as a barrier to TF recruitment (22).

The first evidence of pioneer factors overcoming this barrier came from the observation by DNase I footprinting experiments of FoxA2 and GATA4 binding to their target site in reconstituted and compacted nucleosome arrays (23, 24). To date, several TFs and pioneers were shown to bind nucleosomal DNA *in vitro*, including p53, Oct4, Sox2, Klf4, Zelda, PU.1 or Ascl1 (3, 25, 26, 27). Different binding mechanisms were proposed to account for diverse nucleosome binding patterns. FoxA possesses a winged-helix DNA-binding domain resembling a domain of linker histone H1 and may preferentially target motifs located near the nucleosome dyad, resulting in H1 ejection and increased DNA accessibility (23, 28, 29). Oct4, Sox2 and Klf4 can recognize partial sequences from their consensus binding motif, thereby facilitating the binding of bases exposed on the nucleosome surface (3). Sox2, Oct4 or their heterodimers are sensitive to the position of their binding motif on the nucleosome, efficiently binding at either the nucleosome dyad or extremities and impacting its structure in a way that increases DNA accessibility (30, 31, 32). Other pioneer factors, such as p53 or PU.1, can only recognize their target motif effectively at the extremities of nucleosomal DNA (33, 34), potentially taking advantage of nucleosome dynamics characterized by transient unwrapping at its edges (35, 36). Nucleosome binding was highlighted *in vivo* for some pioneers like Oct4 and Sox2 by observing the colocalization of their binding sites with nucleosomes using the technique of MNase-seq (3), but such colocalization is not seen for all pioneer factors. Finally, nonspecific nucleosome binding was observed for many pioneer factors (3, 33, 37) and this may facilitate nucleosomal DNA binding by increasing their interaction with chromatin. The importance of this interaction is supported by a mutation in a FoxA domain involved in nucleosome interactions that impairs its ability to open compacted nucleosome arrays *in vitro* and to induce chromatin remodeling *in vivo* (38).

The study of Pax7 pioneering kinetics (Fig. 1) revealed that even if it binds its target sites quickly, the subsequent overall increase in DNA accessibility is delayed and more

progressive (6). This and the fact that many bound targets will never be opened highlights that the unique ability of pioneer factors to target condensed chromatin is not automatically reflected by chromatin opening and that these two processes may be mechanistically distinct.

Despite their ability to target condensed chromatin, pioneer factors are only efficiently recruited to a fraction of their potential binding sites genome wide and exhibit lineage-specific binding patterns (7, 10). This could be due to the expression of cell-type-specific partners or the requirement of specific cofactors at some loci. For instance, GATA4 significantly increases FoxA binding to a subset of its potential binding sites (7) and Sox2 recruitment at a subset of its binding sites is dependent on the chromatin-associated protein PARP1 (39). In the case of Pax7, its binding profile in pituitary cells revealed a large subset of so-called Resistant sites where recruitment does not result in any apparent epigenetic change; at another subset, it only leads to enhancer priming and not to the full activation characterized by chromatin opening (6). Collectively, this suggests the existence of various chromatin environments that are more or less permissive to pioneer factor recruitment, stabilization or action. These chromatin environments remain poorly described and are still mainly defined by the absence of epigenetic marks associated with active genes or regulatory elements. Better characterizing them may be paramount to understand the unique ability of pioneer factors to overcome chromatin condensation *in vivo* as well as their mechanisms of action.

Pioneering and epigenetic memory: a one-shot deal!

Cell identity must be stable and maintained as cells divide unless a differentiation process is activated (e.g. through pioneer factor action). At the epigenome level, this inheritance is enacted by mechanisms that maintain chromatin states through cell divisions (reviewed in (40)). Indeed, chromatin organization is maintained after passage of the replication apparatus such that open or closed chromatin are reassembled after replication as they were before replication. In addition to epigenetic marks on histone tails and their associated chromatin components, genomic DNA also bears a modification that is associated with repressed chromatin states, namely methylation of cytosines within the CpG dinucleotide. Maintenance of DNA methylation patterns is critical for the stability of

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cell identity and epigenetic memory is very dependent on this genomic DNA mark.

The purpose of pioneer factors is to activate the expression of a new repertoire of genes, thus implementing a new cell fate. This is achieved through pioneer-driven opening of closed chromatin from an inactive to an active state: that involves a major reorganization of chromatin proteins and their marks, together with changes in DNA methylation. Taken together with ample prior data showing a need for DNA replication to implement cell fate changes, the slow time course of pioneer-driven chromatin opening is consistent with some steps of the pioneering process being dependent on replication.

How could pioneer factors mark their genomic targets during replication fork passage, a process that disrupts chromatin structure and protein–DNA interactions? Indeed, the replication apparatus forces most proteins to dissociate from DNA. However, some proteins are nonetheless able to maintain their position during DNA replication, at least partially, and they are labeled as ‘bookmarking proteins’. These include FoxA1 (41), GATA1 (42) and Sox2 (43), all three being pioneer factors. Interestingly, not all the sites bound by FoxA1 and GATA1 retain these factors, their maintenance seeming to be linked to nucleosome occupancy. We speculate that the retention of pioneer factors on replicating DNA is another of their unique properties.

Globally, chromatin organization is maintained in daughter cells after cell division. According to a recent study, parental nucleosomes marked with repressive epigenetic marks (e.g. H3K9me3) on constitutive heterochromatin are transferred whole to newly synthesized DNA, keeping these PMTs and presumably allowing them to spread on naive adjacent nucleosomes and thus ensuring maintenance of a condensed chromatin structure (44). In contrast, nucleosomes bearing active marks appear to dissociate from DNA at replication, be randomly redistributed after and then re-marked with activating marks. In the context of pioneer action, removal of repressive PMTs may be needed together with the introduction of activating marks for long-term maintenance. Since pioneered sites are surrounded by condensed chromatin, nucleosome marking with activating PMT might not be enough to maintain accessibility and prevent repressive marks spreading. Interestingly, DNA methylation also influences chromatin organization together with histone modifications. There is cross talk between histone modifications and DNA methylation. Some histone PTMs recruit DNA methyltransferases (e.g. ubiquitination of H3 lysines by

Uhrf1 favors the recruitment of Dnmt1, the maintenance methylase (45)), and DNA methylation can indirectly induce some PTMs (e.g. methylated DNA is bound by MeCP2 which recruits Sin3, a complex containing HDAC1/2, histone deacetylases (46)).

DNA methylation at cytosines of the CpG dinucleotides is one of the most extensively studied epigenetic marks. High levels of methylation correlate with transcriptional repression (47) and they are highly linked with condensed chromatin. Genomic DNA methylation follows pre-determined patterns in mammalian development, the first ones being established around embryonic implantation. DNA methylation is performed by DNA methyltransferases (Dnmts), either *de novo* Dnmts (Dnmt3a, Dnmt3b) or Dnmt1 that maintains established DNA methylation patterns. In mammals, CpG dinucleotides are less frequent than statistically expected (48) and their distribution in the genome is not random. Indeed, most CpGs are found at gene promoters and 5′/3′ cis-regulatory regions (49). They often form highly enriched clusters, called CpG islands, that are associated with promoters (in human, around 70% of annotated promoters are within CpG islands (50). In somatic cells, 70–80% of CpG sites are methylated (51). The methylation patterns and their maintenance are vital for correct development, being essential for genomic stability (52, 53), cell differentiation (54), imprinting (55) and transcriptional regulation (47).

Several pioneer factors can trigger DNA demethylation (7, 56). Different mechanisms may be responsible, involving either active DNA demethylation exerted by Ten-eleven-translocation (Tet) enzymes (DNA demethylases) (56) or a passive mechanism involving blockade of DNA methylation maintenance. It is unclear whether DNA (de)methylation is a parallel mechanism of the pioneer action, or whether it is cause or consequence. In the case of FoxA2, the two processes were separated by blocking DNA replication: when the cell cycle is blocked, FoxA2 still opens chromatin but does not trigger DNA demethylation anymore (7). Whether DNA demethylation is a step of the pioneer action or the sole presence of a pioneer factor is sufficient for it to happen is unknown, as well as the putative role of DNA demethylation in the pioneer action. It was however shown that pioneers like Pax7 implement a long-term memory of their action, allowing cells to preserve their gene expression profile when the pioneer is not bound to its pioneered targets anymore. In fact, sites pioneered by Pax7 retain accessibility and active epigenetic histone marks after more than twelve cell passages in the absence of Pax7 (6). DNA demethylation of pioneered enhancers is the likely basis of long-term epigenetic memory.

Early pituitary development and pioneers

Beyond the developmental studies performed during the first half of the twentieth century on pituitary formation, it is mostly the discovery and investigation of TFs controlling pituitary development that has advanced the field. Through a brief review of current knowledge on early pituitary development and cell differentiation (Fig. 3), this section will discuss the putative pioneer function of TFs that are critical for pituitary development. The section will not review the topic of early pituitary development per se as this has been extensively reviewed (57). Since only one TF is clearly shown to exert its role in pituitary development through pioneer action, Pax7 specifying the intermediate lobe melanotrope cells, this section presents a relatively speculative discussion rather than a traditional review of established data.

The pituitary develops from the anterior neural ectoderm that forms the stomodeum and becomes the oral ectoderm. The pituitary primordium, Rathke's pouch, forms at the midline in the back of the stomodeal cavity. The stomodeal ectoderm expresses the related *Pitx1* and *Pitx2* (Pituitary homeobox1/2) genes and these TF's are critical for the progression of Rathke's pouch into the

adult glandular pituitary (58). From the onset of their expression in the stomodeum to fully functioning adult pituitary cells, these TFs are essential, playing a critical role notably in the maintenance of pituitary specific expression of hormone coding genes. Indeed, it is this property that led us to discover the founding member of this family *Pitx1* through its role in control of POMC expression (59). Despite their critical importance, there is as yet no evidence that these factors have pioneer activity and indeed, the limited data available suggest that *Pitx1* does not have heterochromatin interaction properties (6). The *Pitx1/2* genes are required for expression of the downstream *Lhx3/4* TF-coding genes and genetic evidence suggests that the later may fulfill many of the early developmental roles ascribed to *Pitx1/2* in the pituitary, hence the double *Pitx1/2* mutant has similarly arrested pituitary development (at the early pouch stage) as the double *Lhx3/4* mutant (58).

Cells of the pituitary primordium, Rathke's pouch, as well as the pool of adult pituitary stem cells that line the pituitary cleft in the adult gland express the pluripotency pioneer factor Sox2. When these progenitors exit their stem state, they transiently express the related Sox9, but its exact role is not well defined and it is not known

Figure 3

Transcription factor control of cell differentiation during pituitary development. A large body of work from numerous investigators supported the roles of various transcription factors during pituitary cell differentiation; this is reviewed elsewhere (57). Pax7 pioneer activity is required during pituitary development for melanotrope cell specification. In addition, the TFs NeuroD1, MASH1(Ascl1) and GATA2 were shown to have pioneer activity in other systems, but it is not yet clear whether pioneer action of these factors is required for pituitary cell differentiation. The TF Sox2 is a pluripotency factor, it has pioneer activity and it is involved in maintenance of the progenitor state.

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whether it has pioneer activity (60). Sox2 is a pioneer TF and its role in maintenance of pituitary progenitors (Fig. 3) was shown through its tissue-specific knockout (61). The anterior lobe develops through expansion of the ventral part of the early glandular epithelial pituitary and this occurs through an epithelium-mesenchyme transition (EMT) that requires the TF PROP1 (Prophet-of-Pit) (62). PROP1 directly targets a set of genes involved in EMT but whether this involves pioneer action is not known. The earliest cells to reach terminal differentiation in the anterior lobe are the corticotropes driven toward differentiation by the Tbox factor Tpit (63). While Tpit terminates the process of corticotrope differentiation (64, 65), cells destined to this lineage appear to be specified earlier and hence it may not be surprising that Tpit itself does not appear to have pioneer activity (4). It is however noteworthy that Tpit cooperates with the pioneer Pax7 in the related melanotrope cells to establish this cell fate as discussed below (66). Other lineages appear to be specified at a similar time as the corticotrope lineage in the anterior lobe, namely gonadotropes and thyrotropes that express the putative pioneer factor GATA2 at that time but their marker hormone genes are expressed later in development (67, 68). As corticotropes and gonadotropes appear to share a common precursor, it could be that GATA factors (both GATA2 and GATA3 are expressed in the developing pituitary (67)) contribute to a binary switch between these two lineages and this could well be exerted through pioneer action.

Two TF's of the neurogenic bHLH subfamily exert pioneer activity during neuronal development (69, 70, 71). These bHLH TF's, NeuroD1 and Mash1 (Ascl1), are expressed in the developing pituitary with NeuroD1 primarily expressed in corticotropes of the anterior lobe whereas MASH1 is exclusively expressed in the intermediate lobe (72). These factors may thus play a role in pituitary development through pioneer action, but the nature of this role remains poorly defined.

The only pituitary regulatory TF clearly shown to exert its action through pioneer action is Pax7 that specifies the intermediate lobe melanotrope fate and this is discussed below.

Specification of intermediate lobe melanotrope fate Pax7

In the developmental context, the intermediate pituitary is a unique tissue: indeed, it is the only contact point between neural and surface ectoderm that is maintained

throughout life. In early development, this point of contact is a site of intense signaling between neuronal and Rathke's pouch derivatives (57). As the glandular pituitary develops from closure of Rathke's pouch, the primordium of the intermediate lobe is unique by its direct contact with neural tissues and its isolation from the remainder of the developing anterior pituitary by the formation of the progenitor-lined lumen/cleft. As intermediate lobe progenitor cells differentiate, they express Pax7 and this expression overlaps with the expression of the progenitor marker Sox2 (4). Pax7 expression precedes by about half a day expression of the terminal differentiation driver TF Tpit. Whereas knockout of the *Tpit* gene results in blockade of differentiation in both POMC lineages, melanotropes and corticotropes (64), the knockout of *Pax7* does not in itself prevent differentiation but Tpit expression in absence of Pax7 leads to differentiation into corticotropes (4, 66). Pax7 is thus the requisite binary switch for melanotrope differentiation but not for differentiation *per se*. It is nonetheless interesting that Pax7 was found to regulate expression of the cell cycle inhibitor that controls progenitor cell cycle exit, p57^{Kip2} (4).

Overexpression of Pax7 in a pituitary-wide gain-of-function transgenic experiment was only found to be incompatible with the gonadotrope fate (4). We may speculate that this incompatibility is related to early GATA2 expression in cells committed to the gonadotrope lineage: could the pioneer actions of GATA2 and Pax7 be antagonistic?

Pax7 and the melanotrope program of gene expression: an affair of enhancers, not promoters

The implementation of the melanotrope cell fate by Pax7 requires its pioneer action. Genome-wide this is achieved through chromatin opening triggered by Pax7 binding to about 2000 enhancers (4, 6). This set of melanotrope-specific enhancers were initially identified by expressing Pax7 in the model corticotrope cell line AtT-20, and thus transdifferentiating them into melanotrope-like cells (Fig. 2C). Their relevance was confirmed by comparison of genomic accessibility profiles in normal mouse melanotropes compared to corticotropes (66). The genomic landscapes of these normal mouse cells were compared using the ATACseq technique that reveals accessible DNA at active regulatory elements such as promoters and enhancers (Fig. 2B). Comparison of ATACseq landscapes for these lineages as well as with

other cells of the mouse pituitary revealed that cell-specific regulatory elements are differentially accessible enhancers and that very few promoters show cell-restricted accessibility (6). For the POMC lineages, only about 20 promoters exhibit cell-specific accessibility by ATAC and these include the promoters of genes for cell-specific regulators. For example, the *Pax7* promoter is not accessible in corticotropes, whereas the *NeuroD1* promoter is not accessible in melanotropes (66). This contrasts with about 250 genes that are uniquely expressed in either corticotropes or melanotropes and for which the bulk of promoters are accessible in both lineages. Differential expression of these genes therefore appears to depend on differentially accessible enhancers in each lineage (6, 66).

Comparison of the genomic landscapes in melanotropes and corticotropes further reveals that it is not only enhancers, but very often entire genomic domains known as topologically associated domains (TADs) (73) that exhibit marks of activity in melanotropes but not in corticotropes. For example, the entire TADs encompassing the genes of the melanotrope-specific Protein convertase 2 (*Pcsk2*) as well as that containing the dopamine receptor 2 (*Drd2*) gene are in active/open conformation in melanotropes but not in corticotropes. This suggests that the action of Pax7 in the melanotrope lineage not only acts locally to open Pax7-dependent enhancers but also more globally to regulate the accessibility of entire TADs that cover hundreds of thousand base pairs, containing many genes and regulatory elements (66). The mechanism responsible for this Pax7-dependent opening of entire TAD domains remains completely elusive. It is also unclear whether this process bears any similarity to the large-scale chromatin remodeling action of pluripotency factors.

We have a better understanding of local changes that occur at Pax7-dependent enhancers (6). Before Pax7 action, the chromatin of these melanotrope-specific enhancers harbours repressive marks typical of facultative heterochromatin such as H3K9me2 and the enhancers are not accessible to nonpioneer factors such as Tpit. Pax7 is rapidly recruited to these sites but this initial binding is weak (Fig. 1). Over a period of 12 to 24h, Pax7 binding is stabilized and this is accompanied by deposition of active enhancer chromatin marks such as H3K4me1. This stabilisation is accompanied by progressive opening of the chromatin as revealed by ATACseq. Evidence of transcriptional activity at these enhancers is provided by the recruitment of p300 and the deposition of its mark, H3K27ac. Finally, transcriptional activation of the genes targeted by these enhancers occurs over the course of a few days. This is thus a lengthy process by comparison to

simple transcriptional activation triggered by TF binding to already opened enhancers as this usually occurs within hours.

Both the differentially accessible regions in normal melanotrope pituitary cells and the Pax7-dependent activation of enhancers in the model AtT-20 cells identified about 2000 enhancers that constitute the bulk of the melanotrope-specific regulatory subset. It is however interesting that in AtT-20 cells, Pax7 was also found to act at another subset about 8000 putative enhancers that switch from an inactive to a primed state following Pax7 pioneering. This primed state may represent an intermediate state in the process of complete enhancer activation (Fig. 1). It is characterized by the presence of the enhancer mark H3K4me1 with low, if significant, ATACseq/DNA accessibility signal in absence of p300 or H3K27ac. This priming process may constitute the initial chromatin opening, that is the hallmark of pioneer action, and prepare these enhancers for further activation through recruitment of other nonpioneer TFs.

Cooperation between pioneer and nonpioneer TFs Pax7 and Tpit

The dependence on both Pax7 and Tpit for melanotrope differentiation allowed us to query the role of each of these factors, the pioneer Pax7 and the nonpioneer Tpit, in the process of chromatin opening (Fig. 4). It was first surprising to realize that both factors are required for chromatin opening at the melanotrope repertoire of enhancers as Tpit did not appear to have pioneer activity. Indeed, melanotrope-specific enhancers as well as entire melanotrope-specific TADs (such as *Pcsk2* and *Drd2* TADs) failed to open in both Pax7 and Tpit knockout intermediate lobes as revealed by ATACseq (66). The reconstitution of Pax7-dependent chromatin opening in the Tpit-expressing AtT-20 cells compared to the Tpit-negative α T3 cells supported the conclusion that Tpit is required for bulk chromatin opening as detected by significant ATACseq signal gains. In contrast, Pax7 action in Tpit-deficient cells only leads to modest ATACseq signals commensurate with the early or first phase of pioneer action. These observations are important in that they indicate that the unique property of Pax7 (and presumably other pioneers) is to recognize targets in closed heterochromatin and to initiate an initial, and relatively subtle, change in chromatin organization. In pituitary melanotrope cells, the next steps in enhancer opening initiated by Pax7 are dependent on Tpit as it is

Figure 4

Cooperation between the pioneer Pax7 and the nonpioneer Tpit for establishment of the melanotrope cell fate. In normal development, the nonpioneer TF Tpit determines the corticotrope cell fate in the anterior pituitary and knockout of the *Tpit* gene abrogates this differentiation (64). In the intermediate pituitary, expression of the pioneer Pax7 is required for chromatin opening at a set of about 2000 melanotrope-specific enhancers and a large proportion (about 70%) of these enhancers recruit the nonpioneer Tpit following Pax7 action (4, 6). The essential role of Pax7 pioneering is supported by the phenotype of the *Pax7* knockout intermediate pituitary cells that completely switch to a corticotrope fate in absence of Pax7. Analysis of chromatin states in intermediate lobes of *Pax7* and *Tpit* knockout pituitaries showed the essential cooperation between the pioneer Pax7 and the nonpioneer Tpit for establishment of the melanotrope-specific chromatin landscape (66).

co-recruited to the same enhancers through its cognate DNA sequence and through protein/protein interactions with Pax7 (4, 66). This involves recruitment of chromatin remodeling complexes such as the Swi/Snf complex leading to nucleosome eviction. Although a very large proportion of Pax7-dependent pioneered enhancers also recruit Tpit in melanotrope cells, some Pax7-pioneered enhancers do not show Tpit recruitment and hence, it is envisioned that other TFs may fulfill a similar role as Tpit in these instances.

Similar relationships between pioneer and nonpioneer TFs may be involved in the action of other pioneers. For example, the action of steroid hormone receptors is often dependent on prior priming by the pioneer FoxA: this is discussed below in the context of hormone-dependent cancers. In conclusion, recruitment of most TFs leads to enhanced chromatin opening but the critical action of pioneers is not *per se* linked to bulk chromatin opening. Rather, the unique properties of pioneers is to recognized targets in closed heterochromatin and perform an initial, and relatively subtle, chromatin remodeling that is still unresolved in nature.

Pioneers in tumorigenesis

As the normal function of pioneers is to implement new cell fates and gene expression programs, their dysregulation has dire consequences. In fact, many pioneers were linked to cancers, such as Sox2 in squamous cell cancers (74), Pax7/Pax3 in rhabdomyosarcomas (75), and FoxA1 in hormone-dependent cancers (76). While the implication of pioneers in tumorigenesis seems clear, causative links or precise mechanisms are mostly lacking.

Some pioneers regulate cell differentiation while others maintain the stem cell state. The pluripotency factor Sox2 is critical for stem cell renewal and maintenance of pluripotency (77). Stem cells, as well as cancer stem cells, have unique pathways controlling self-renewal, proliferation and differentiation (78). With regards to Sox2, genomic amplification of the *Sox2* gene leading to increased levels of Sox2 protein was found in ~20% of lung esophageal squamous cell carcinomas whereas ectopic expression of Sox2 increases tumor cell self-renewal *in vitro*, and knockdown of Sox2 expression decreases tumor proliferation and anchorage-independent growth (74,

79). Further, *in vivo* knockdown of Sox2 prevented tumor growth initiation (80, 81). While these studies support the idea that high Sox2 expression may correlate with poor outcome, this may not be true in all cases (82). In spite of Sox2 expression marking pituitary progenitors, its (mis)expression is not associated with pituitary tumor development. However, rare Sox2 gene mutations affect hypothalamo-pituitary development (83).

Pax7 specifies the melanotrope fate during pituitary development and this is accompanied by widespread changes in gene expression (66), including a switch in the control of cell cycle re-entry from p57^{Kip2} to p27^{Kip1} (4, 84) in parallel with the switch between Sox2 and Pax7 expression. This switch may be the direct consequence of Sox2 repression by p27^{Kip1} (85). While p27^{Kip1} expression is lost in many pituitary adenomas, in particular Cushing's, and knockout of the p27^{Kip1} gene in mice leads to pituitary tumor development (86), there is as yet no evidence of Pax7 involvement in these adenomas. Mutations of the p27^{Kip1} gene itself cause the multiple endocrine neoplasia type 4 (MEN4) syndrome (87, 88).

While Pax7 has not yet been involved in pituitary tumors, it has in myogenic tumors. Indeed, Pax7 is critical for myogenic development and its expression is maintained in adult muscle satellite cells (89). Satellite cells are adult muscle stem cells that are activated in response to muscle injury. Pax7 is required to maintain the satellite cell pool by promoting cell survival and blocking cell differentiation, allowing long-term muscle renewal (90). Without Pax7, cells enter terminal differentiation and the cell cycle is blocked (91). Abnormal or untimely expression of Pax7 may, therefore, promote tumorigenesis (92). And indeed, Pax7 is expressed in most rhabdomyosarcomas: one study found that 83% of embryonal rhabdomyosarcomas are positive for Pax7 (93).

Further, many rhabdomyosarcomas have chromosomal translocations involving Pax7 or the related Pax3 (94). These translocations involve the DNA binding domains of Pax7 or Pax3 fused to the transactivation domain of FOXO1 in a majority of alveolar rhabdomyosarcomas (15% and 52% respectively in older patients) (95). These translocations create a new TF that stimulates cell proliferation, promotes cell survival, blocks terminal differentiation and promotes cell migration and invasion (96).

Hormone-dependent cancers, including breast and prostate cancers, are some of the most preponderant cancers (97). Most are driven by nuclear hormone receptors, namely estrogen receptor (ER) and androgen receptor (AR). ER and AR activate the transcription of

breast- and prostate-specific genes upon binding of their natural ligand. In the last decade, some mechanisms by which these tumors grow, and progress have been identified, including the significant role of FoxA1-GATA2 and FoxA1-GATA3 in regulating AR and ER transcriptional activity, respectively (98). FoxA1 is part of the FoxA family, containing FoxA1, FoxA2 and FoxA3, that are pioneer factors driving notably prostate, mammary gland, pancreas, liver and lung differentiation (1, 99). FoxA1 is also required for hormone receptor activity, enabling their binding to enhancer sequences (100, 101, 102). These sites are also often enriched in GATA motifs. Thus, pioneer GATA TFs appear to cooperate with FoxA1 to recruit ER/AR to their target sites and enhance gene transcription (98). The specific mechanisms linking FoxA1, GATA TFs and hormone-dependent cancers still require clarifications. Nonetheless, genomic amplification of the FoxA1 locus is found in several cancers leading to FoxA1 overexpression, and the gene itself is mutated in about 1.8% of breast cancer and 3 to 5% in prostate cancer (76). Further, FoxA1 overexpression is linked to poor prognosis and it appears to be linked to endocrine therapy resistance (103).

Another pioneer factor, C/EBP α , drives myeloid cells differentiation and induces cell cycle arrest in several myeloid lineages (104). It was suggested to act as a tumor suppressor because loss-of-function mutations in the C/EBP α gene trigger development of acute myeloid leukemia (AML) and C/EBP α gene activity is lost in ~10% of AML cases.

In summary, whether it is indirectly by promoting differentiation or by directly regulating cell growth pathways, pioneer misexpression is often associated with cancer development. These critical and unique TFs have pivotal roles as master regulators of basic cell functions: hence, we need to understand the mechanisms underlying their pleiotropic actions on gene regulatory pathways in normal development in order to comprehend and master their damaging effects in cancer.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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