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Manipulation of Gene Expression in Megakaryocytes

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1. Introduction

The process of megakaryocyte generation in the bone marrow and subsequent differentiation leading to platelet production is little understood. Known as *megakaryopoiesis*, it involves a number of unique biological features, including an increase in the nuclear DNA content (endoreplication) and partitioning of cytoplasm and membranes into platelets. Abnormalities of thrombosis and hemostasis can occur as a result of alterations in the number of platelets generated and maintained in the circulation or through aberrations in the functional behavior of the platelet itself. Although some of these abnormalities are most likely indirect in their etiology (for example, immune thrombocytopenias), many are directly linked to an inherited or acquired genetic effect operating at some point during megakaryopoiesis. Inherited mutations affecting megakaryocytes and platelets are relatively rare, although there is increasing interest in the association between genetic polymorphisms in platelet proteins that have no profound phenotype but may be linked to an increased thrombotic risk. Specific genetic changes leading to deregulation of proliferation and/or differentiation at some point during megakaryopoiesis are also implicated in several acquired conditions, including leukemias, preleukemic states, and dysplasias.

Experimentation directed at understanding normal or disease-related biological processes has been restricted in different ways for megakaryocytes and platelets. Classically, studies of megakaryocyte function have involved work with cell lines that have features in common with megakaryoblasts and can often be induced to undergo some aspects of terminal differentiation. Although such cells lend themselves to manipulation, they are immortalized, often deriving from leukemic patients, and therefore have aberrant proliferation and differentiation characteristics. Although it is preferable to use primary cells, the scarcity of megakaryocytic cells in the bone marrow has limited their usage. In recent years, improvements in cell purification and culture conditions, especially through use of the specific growth factor thrombopoietin, has increased the range of research that can be conducted ex vivo. The study of abnormal human

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megakaryopoiesis is further restricted either because of the small number of affected individuals or because it is impractical or clinically undesirable to collect bone marrow. Although such work has been, and will continue to be, of great value, it would also be advantageous to be able to study normal or mutated gene products in an in vivo context. Studies on platelets have been less affected by restraints on material availability, except in relation to that deriving from patients with rare specific genetic defects. Biochemical approaches aimed at examining affector and effector responses and the signaling pathways linking the two are well worked out. The major limitation, however, is with respect to the manipulation of gene products within the platelet. This is most obviously a consequence of the absence of nuclear DNA, and the difficulties associated with expression of exogenous proteins.

The clearest solution to many of these limitations is to work with cells derived from individuals or animals carrying specific genetic changes in the proteins of interest. Some, but not all, genetic diseases affecting human megakaryopoiesis or platelet function have been linked to mutation in a specific gene. In a few cases a corresponding spontaneous mutant mouse strain has been identified. However, most genes of interest to those studying megakaryopoiesis and platelet function do not have a naturally occurring mutant form in either humans or mice.

In this perspective we will highlight the advantages of using the mouse for the investigation of megakaryocyte and platelet biology through modification or ablation of a gene product of interest although some of the methods, especially those used on cells ex vivo, are equally suited to work with human cells. Broadly speaking the discussion will be divided into two areas: (1) introduction of exogenous genetic material, and (2) ablation or modification of endogenous genes. Within each of these areas we will consider techniques which are suited to either ex vivo or in vivo studies and we will attempt to speculate on future directions.

2. Introduction of Exogenous Material

Several strategies are available for the expression of exogenous proteins in cells of the megakaryocyte lineage, although the possibilities for applying such methods directly to platelets are more limited. These methods can be categorized into those suitable for ex vivo use and those that enable exogenous gene expression in vivo. Methods utilized ex vivo are largely dependent on purification and expansion of megakaryocytic cells, usually bone marrow- or umbilical cord-derived, or cells that can become committed to megakaryopoiesis under appropriate culture conditions. Such cell enrichment protocols are outside the scope of this chapter and the reader is instead referred to recent reviews on the topic ***(1)***. Of the three principal routes for introducing exogenous material, the most commonly used is infection with viral vectors. Transfection of expression vectors, although strictly speaking a possible means of introducing genes of interest, is not generally considered for work with primary megakaryocytes and will not be discussed here. Recently, though, there has been considerable interest in the prospect of direct introduction of proteins as fusions to small basic peptide sequences, and this method will be considered in particular because of its potential for use with platelets as well as megakaryocytes. Expression of exogenous sequences in vivo can also be

achieved through viral infection; however, there are considerable difficulties with respect to the efficiency and specificity of this route (*see* **Subheading 2.1.3.** for a possible way around some of these limitations). The generic method of choice for in vivo expression of protein-coding sequences in mice is transgenesis, and a number of permutations will be discussed, especially in regard to how expression of the exogenous gene product can be limited to megakaryocytes and platelets.

2.1. Infection

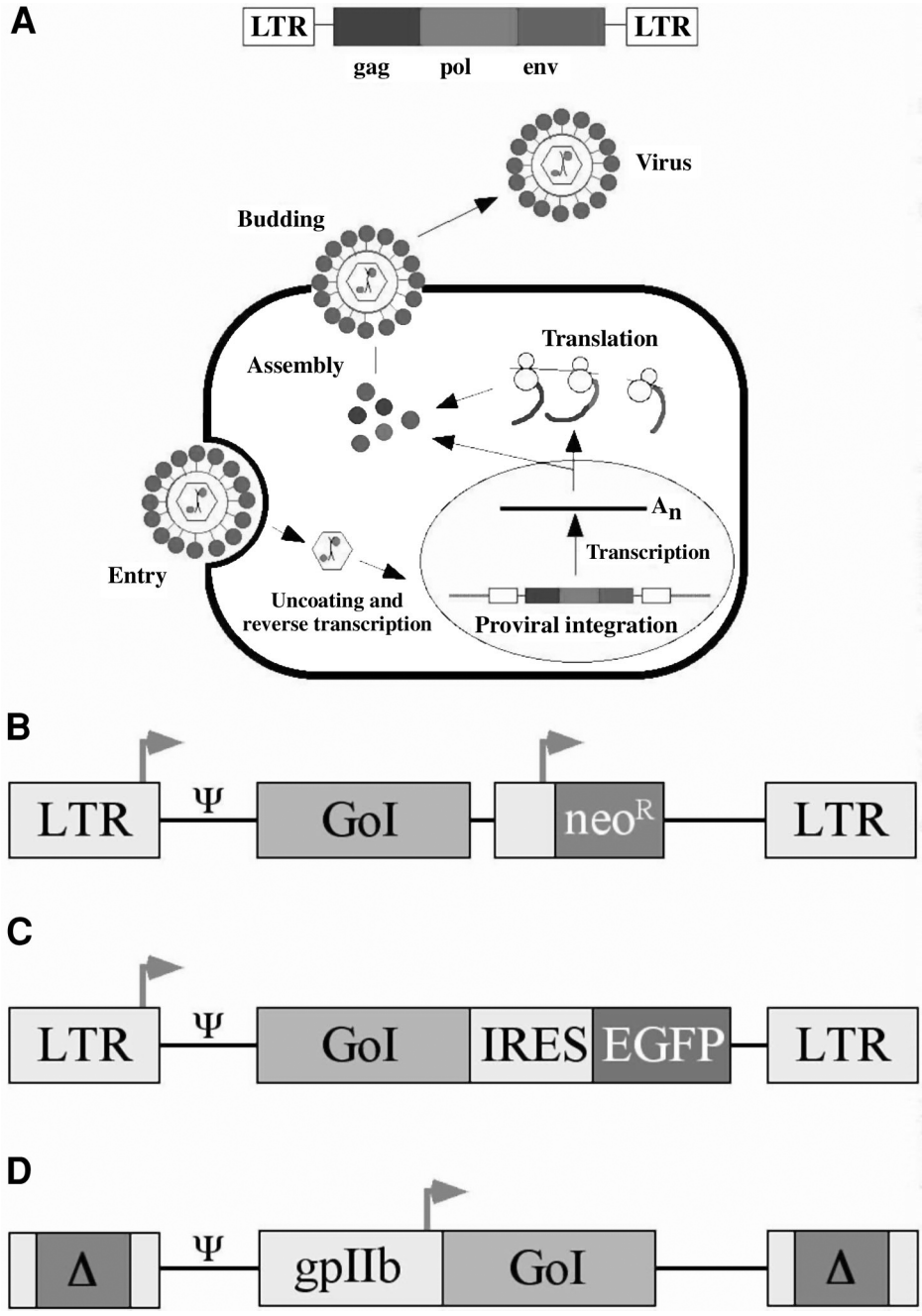
Vector systems derived from retroviruses, lentiviruses, adenoviruses, and herpes-related viruses are available for use. None of these viruses has an inherent capacity for restricted expression in megakaryocytic cells; however, as suggested above, "selectivity" can be achieved by expansion of the desired cells in culture prior to infection. A few (as yet little-used) alternative means will be discussed that can be used to obtain megakaryocyte-specific infection or expression from a viral vector.

2.1.1. Retroviral Infection

Delivery of exogenous sequences as retroviruses has the advantage that not only can a large proportion of cells be infected, but also the conversion of the retroviral RNA genome into an integrated DNA intermediate (the provirus) results in permanent genetic modification **(Fig. 1A)**. Classical retroviral vectors usually yield proviruses that constitutively express the exogenous gene from the viral LTR promoter/enhancer sequences **(Fig. 1B)**. This relatively simple approach has been used effectively to infect $CD34^+$ progenitors derived from human peripheral blood or bone marrow. For example, Burstein et al. ***(2)*** were able to culture infected progenitors under conditions favoring megakaryocyte development and demonstrate that 60% of the platelets contained the exogenous gene product. The murine stem cell virus (MSCV, ***[3]***) is ideal for infection of mouse hemopoietic cells and is frequently used in a form that co-expresses the exogenous gene of interest together with GFP to enable tracking of those cells that have been infected. Such a viral vector (MIGR, **Fig. 1C**) was used, for example, by Baccini et al. ***(4)*** to introduce the cell-cycle inhibitor p21 into ex vivo cultured megakaryocytes.

2.1.2. Regulation of Retroviral Gene Expression

If it is desirable to infect megakaryocytic cells selectively in a mixed population (perhaps to avoid preselection and culturing) or to restrict expression to a defined stage of differentiation, then one possible solution is to use retroviral vectors that contain an internal promoter **(Fig. 1D)**. Full effectiveness of the specificity of the internal promoter is ensured by an additional modification to the vector in the form of a deletion of the enhancer sequences from the 3′LTR. The mechanism of proviral integration of such so-called self-inactivating (SIN) retroviruses results in no LTR-driven transcription. That this approach can be utilized to restrict expression to megakaryocytes was shown by Wilcox et al. ***(5)***, who infected human $CD34^+$ cells with a retrovirus in which the protein of interest was driven from approx 900 bp of the promoter of the *gpIIb* (α_{IIb} integrin) gene. Increasing understanding of megakaryocyte-specific


A
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GoI
neoR
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C
LTR
Ψ
GoI
IRES
EGFP
LTR
D
Δ
Ψ
gpIIb
GoI
Δ

gene regulation may enable this approach to be extended if it is possible to identify promoter sequences that act during particular stages of megakaryocyte differentiation **(Table 1)**.

2.1.3. Targeting of Retroviral Infection

Unlike their mammalian counterparts, avian retroviruses cannot infect and replicate in murine cells due to the lack of a cell surface receptor and incomplete intracellular virus assembly ***(6)***. However, engineered expression of the subgroup A avian leukosis virus (ALV-A) receptor, TVA, on the surface of murine cells confers susceptibility to infection by ALV-A viruses and by murine retroviruses packaged with the ALV-A envelope protein (EnvA). By expressing TVA from a transgene (*see* **Subheading 2.3.**) driven by megakaryocyte-specific promoters, it is possible to restrict retroviral infection (**Fig. 2** and ***[7]***). Both avian (e.g., RCAS, ***[6]***) and murine (e.g., MuLV- or MSCV-based) vectors can be employed, although the latter have to be packaged into virions containing the EnvA protein ("pseudotyping") and cannot generally be produced in such high titers as the avian viruses.

In addition to the specificity of infection, this system has two other major advantages. First, multiple sequential infections can be performed on the target cells using

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Fig. 1. *(see facing page)* **(A)** Schematic representation of basic retroviral structure and the process of infection. A retroviral genome consists of a single-stranded RNA molecule encoding the viral core proteins (gag), reverse transcriptase (pol) and the envelope glycoprotein (env). These genes are flanked by sequences (LTRs—long terminal repeats) involved in the conversion of the RNA genome into a double-stranded DNA form allowing integration into the host genome and subsequent transcription from the resultant so-called provirus. Retroviral vectors are modified versions of this basic structure, in which coding sequences for the gene of interest (GoI) are inserted between the LTRs, allowing their stable integration into the genome. Such vectors are generally unable to provide all the components for retroviral infection because of removal of all or part of the gag, pol, and env genes (replication-incompetent). In this case, packaging of the vector genome into viral particles is achieved by supplying these gene products on a "helper" retrovirus or through use of a "packaging" cell line. Many variations can be made to the basic retroviral structure to generate a suitable vector. The simplest type **(B)** contains the gene of interest which is transcribed from the LTR promoter elements and a selection cassette (e.g., neoR) driven by a second internal promoter (usually a strong constitutive promoter such as that from the PGK gene or the SV40 virus). It is often desirable to track infected cells; this can be achieved by co-expression of a fluorescent protein. In the case illustrated (**C**, the so-called MIGR vector), the fluorescent protein (EGFP) is produced from a bicistronic RNA through use of an internal ribosome entry site (IRES). If it is desirable to restrict expression of the gene of interest, one possible solution is to use a lineage-specific internal promoter, such as that from the *gpIIb* gene **(D)**. In this case, a deletion is included in the LTR sequences to prevent these from driving strong constitutive expression. (*See* color insert following p. 300.)

Table 1
Examples of Genes Expressed Predominantly or Exclusively in the MK Lineage

Gene	Other tissues where expression has been detected	Used to drive transgene expression
gpIbα	Endothelium	+
gpIbβ	Endothelium	
gpIIb	Hemopoietic progenitors, mast cells	+
gpV	Endothelium	
gpVI		
gpIX	Endothelium	+
von Willebrand factor	Endothelium	
P-selectin		
PCLP1 (thrombomucin)	Endothelium, kidney podocytes	
AA4.1	Endothelium, lung epithelium	
c-Mpl	Hemopoietic progenitors	+
PF4		+
PBP		
β1 tubulin		

viruses encoding different genes. This is possible because, unlike infections with mammalian viruses, mouse cells infected with EnvA-packaged retroviruses do not express sufficient EnvA to elicit the phenomenon of resistance to superinfection ***(6)***. Second, a single transgenic mouse strain can be used as a means to introduce many different genes of interest, thereby avoiding the need to generate transgenic lines for each gene product or variant to be analysed. TVA expression driven by *gpIbα* or *gpIIb* gene promoter elements has been used very effectively to reconstitute protein expression in megakaryocytes derived from both the NF-E2 and c-*mpl* knockout mice (***8*** and ***9***, respectively). Future extensions of this methodology will doubtless include the generation of TVA-expressing strains in which the transgene is driven by alternative megakaryocyte-specific promoters. Indeed, additional strains expressing TVA in megakaryocytes have been created using promoters from the *PF4*, *gpIX*, and c-*mpl* genes (G. J. M. and Andrew Leavitt, unpublished). The possibility of infection and exogenous gene expression in progenitors prior to their commitment to megakaryopoiesis has also been made possible with a mouse strain in which TVA is expressed from the SCL gene 3′ enhancer ***(9a)***.

2.1.4. Lentiviruses as a Means of Infecting Nonreplicating Cells

Retroviruses fail to integrate in nonreplicating target cells due to a block that occurs before entry into the nucleus of the infected cell. This problem may be relevant to the targeting of megakaryocytic cells once they have commenced endoreplication. Hence, it is known that simple retroviruses are not effective as a means of transducing mature

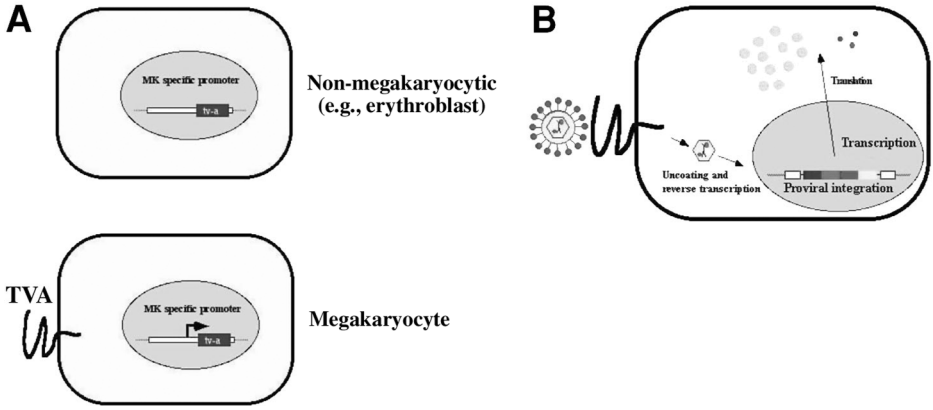

Fig. 2. Megakaryocyte-specific retroviral infection through the TVA receptor. **(A)** The *tv-a* transgene is transcribed from a specific promoter so that the TVA receptor is only expressed on the surface of megakaryocytic cells. **(B)** Retroviral particles coated with the EnvA glycoprotein bind to TVA, allowing viral entry and proviral integration. Following LTR-driven transcription, the protein encoded by the gene of interest (yellow) is expressed efficiently compared to the avian retroviral proteins *gag* (blue), *pol* (red), and *env* (green). (*See* color insert following p. 300.)

megakaryocytes, but it is unclear whether this is due to an absence of nuclear membrane breakdown that may be associated with endoreplication. However, as for other nonreplicating cells, this limitation can be overcome through use of lentiviral vectors ***(10)***. The relatedness of lentiviruses to retroviruses allows for the possibility of using them in combination with the TVA system to facilitate entry into megakaryocytic cells. Pseudotyping of a modified human immunodeficiency virus-based lentiviral vector with EnvA has been shown to be an effective way of using lentiviral vectors in conjunction with TVA systems ***(11)***. Indeed, recently we have been able to utilize a feline immunodeficiency virus-based lentiviral vector in a similar way to efficiently infect fully mature polyploid megakaryocytes (G. J. M., J. F., and Andrew Leavitt, unpublished observations).

2.1.5. Other Viral Delivery Systems

Sindbis viral vectors have been utilized once, to our knowledge, to provide transient gene expression in terminally differentiated megakaryocytes ***(8)***. A much-investigated and exploited vector for transfer of exogenous sequences to mammalian cells, particularly for human gene therapy, is based on adenovirus. The feasibility of using recombinant adenoviruses to infect megakaryocytes has been shown, for example, by Faraday et al. ***(12)***, although there seems to be no obvious advantage to match the efficiency, specificity, and heritable integration that can be achieved with retroviral and lentiviral vectors. Finally, if it is necessary to introduce very large segments of

DNA (20–100 kbp), then herpes simplex virus type I (HSV-1) could be a possible solution (*see*, for example, **ref. *13***), although we know of no examples yet of application to studies on megakaryocytes.

2.2. Direct Introduction of Protein

Recently, protein transduction has been shown to be a highly efficient method to introduce proteins into mammalian cells ***(14)***. By fusing a basic 11-amino-acid peptide derived from HIV-TAT to a protein of interest, it is possible to render it into a cell-permeable form. A wide variety of cell types are able to incorporate the fusion proteins, although there is no specific description yet of the efficiency of entry into megakaryocytic cells. However, this method clearly should have useful applications in the study of megakaryocytes and it will be interesting to determine whether platelets are also amenable to protein uptake.

2.3. Transgenesis

Transgenesis involves the integration of copies of DNA sequences encoding the gene of interest (the "transgene") randomly into the genome. These genetic modifications are inherited and can be bred into appropriate genetic backgrounds. The number of copies may vary widely and the site of integration can determine the specificity and level of transgene expression. The basic method of transgenesis involves injection of the transgene DNA into 2-d-old embryos that are then transplanted back into a pseudo-pregnant female. Live pups (potential founders) can then be screened by Southern blotting or PCR of a small sample from the tip of the tail to determine the presence and copy number of integrated DNA ***(15,16)***. The essential features of the transgene are a promoter, an intron element to mimic normal gene structure, the coding sequences of the molecule under investigation, and a polyadenylation signal **(Fig. 3A)**.

2.3.1. Conventional Transgenes

The choice of promoter used to drive expression of the sequences encoded in the transgene is of crucial importance. Promoters that give rise to widespread expression of the exogenous gene are of limited use because of the likelihood of problems associated with inappropriate expression. Usually, the transgene is designed so that it should be expressed in specific cell types. Limiting expression of transgenes to the megakaryocyte lineage can be achieved by harnessing the control elements of genes that are exclusively, or at least predominantly, expressed in megakaryocytes. Examples of gene promoters that have been, or have the potential to be, used in this way are listed in **Table 1**.

2.3.2. Regulated Expression from a Transgene

An alternative way of limiting transgene expression is to make use of a promoter that is able to be expressed in all cell types but is under the control of a regulator. The activity of the regulator is controlled by the in vivo administration of a small molecule that can be taken up by all cells. There are a number of systems of this type (for review, *see* **ref. *17***), but by far the most widely exploited one makes use of the tetracycline-dependent

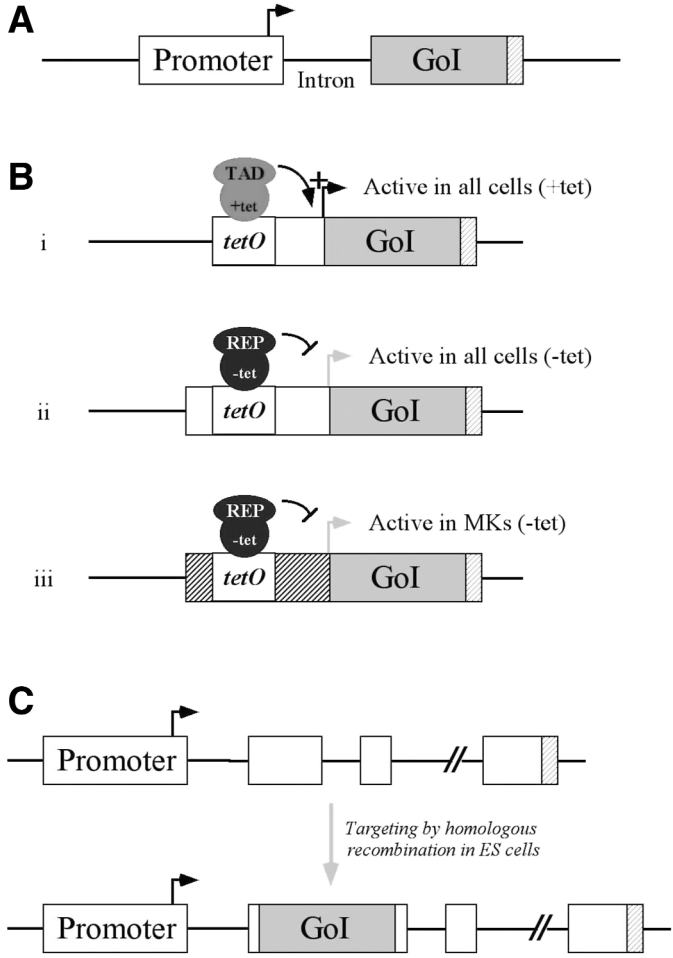

Fig. 3. Some strategies for transgenic expression of the gene of interest. **(A)** A conventional transgene consists of a promoter and the coding sequences (GoI) separated by a small intron. A polyadenylation signal sequence (hatched box) ensures correct transcriptional termination. **(B)** Tetracyline (tet)-regulated systems for the control of transgene expression. In all examples shown, the tetracycline-dependent regulator binds to its recognition motif *(tetO)* either in the presence *(i)* or absence (*ii* and *iii*) of tetracycline. In *(i)* the promoter element is essentially inactive until tet binding to the regulator brings the transcription activation domain (TAD) into proximity of the initiation site (arrow). In contrast, in *(ii)* and *(iii)* the promoter driving the transgene is dominantly repressed by a repressor domain (REP) linked to the regulator. When tetracycline is added, the regulator no longer binds to *tetO* and the gene of interest can be transcribed from either a constitutive *(ii)* or a megakaryocyte-specific promoter *(iii)*. **(C)** Precise lineage and stage-specific expression can often be achieved by introduction of the gene of interest into the genomic locus of a gene which has the desired expression pattern. In the example shown, targeted insertion is into a coding exon.

reversal of the binding of the bacterial tet repressor (TetR) to its DNA-recognition motif, the tet operator (*tetO*, ***[18]***). Although there are several possible permutations of this system using different engineered variants of TetR ***(19)***, we will consider three basic strategies that could be employed in megakaryocytes **(Fig. 3B)**. Fusion of TetR to the transactivation domain of HSV-1 VP16 creates a transactivator (tTA) that binds *tetO* in the absence of tetracycline ***(18)***. Wider applicability in vivo has been achieved by mutation of tTA so that it binds to *tetO* only in the presence of tetracycline. Therefore, by linking the gene of interest to an essentially inactive promoter element containing multimers of *tetO*, a transgene is created that should be expressed only when cells encounter tetracycline in vivo or ex vivo **(Fig. 3Bi)**. However, there are two drawbacks to this approach. First, induced expression will be in all cell types, perhaps leading to some of the same problems that might be encountered with a constitutive transgene. Second, it is difficult to limit the basal expression of the transgene in the absence of tetracycline. An alternative, but as yet little-utilized, strategy involves fusion of TetR to a dominant suppressor domain (tTS, ***[20]***). tTS will dominantly repress expression from a promoter containing *tetO* unless tetracycline is added to bring about its removal from DNA **(Fig. 3Bii)**. In the context of restricting transgene expression to megakaryocytes, it is likely that *tetO* could be incorporated into a transgene driven by a megakaryocyte-lineage-specific promoter. If this transgenic line is bred together with a strain expressing tTS from a constitutive promoter, then megakaryocytic cells in the resultant offspring will express the gene of interest only when the animals are fed tetracycline **(Fig. 3Biii)**.

2.3.3. Knock-In

The random integration of multiple copies of transgenic DNA into the genome often leads to expression that is not ideal, either in terms of cell type restriction or the level of transcription. A possible solution is to insert the transgenic cassette directly into a gene locus that is expressed specifically or predominantly in megakaryocytes **(Fig. 3C)**. This so-called knock-in approach involves gene targeting in mouse embryonic stem (ES) cells. Practically, this is achieved by generating a targeting vector that consists of DNA isogenic with the ES cells to be modified and designed to carry the required modifications as well as flanking arms of unmodified DNA that are necessary for homologous recombination ***(21)***. The vector bears a constitutively active antibiotic resistance gene (usually neo^R conferring resistance to G418) for selection of targeted cells and a negative selection cassette (usually the HSV-1 *tk* gene conferring sensitivity to gangcyclovir) for elimination of those integration events that have occurred through nonhomologous recombination **(Fig. 3C)**. The targeting vector is introduced into ES cells by transfection and positive and negative selections are applied. Potential clones are expanded and analyzed by Southern blotting and PCR to determine whether homologous recombination has occurred. A correctly targeted ES clone is then injected into blastocysts, which, after reimplantation, develop to produce a chimeric founder. If the ES clone has retained totipotent potential and contributes significantly to the chimera, then the modified gene should be transferable to the next generation and a line will have been created (*see* **Fig. 6**). This is a labor-intensive strategy and should perhaps be undertaken only if transgenesis using a specific promoter fails. Another

possible disadvantage is that the sequences being "knocked in" can sufficiently disrupt the target gene so that a null allele is generated. In this case modified animals could only be used as heterozygotes. An example of such a situation is described by Tronick-LeRoux et al. ***(22)*** who inserted a thymidine kinase transgene into the *gpIIb* gene.

3. Gene Ablation or Modification

3.1. mRNA Ablation

A number of methods exist for reducing the level of mRNAs that could be applied to expanded and purified megakaryocytes ex vivo, although there are few examples in the literature of their use on such cells. The common theme to these techniques is that they rely on RNA or DNA sequence complementarity to the target mRNA as a means to interrupt its translation or to initiate its destruction.

3.1.1. Antisense

The simplest approach of this type involves the use of a short single-strand oligonucleotide that, upon formation of a hybrid RNA:DNA or RNA:RNA duplex, initiates destruction of the mRNA by recruitment of RNAseH. Delivery need not be directly in the form of an oligonucleotide but can be as RNA transcribed from an introduced expression construct or virus (*see* **Subheading 2.1.**) or from a transgene (*see* **Subheading 2.3.**). One adaptation to the use of oligonucleotides, which is not amenable to introduction on a vector but which has become popular recently, uses synthetic DNA analogs called morpholino phosphorodiamidates. Morpholinos, as they are usually known, have highly favorable properties and are being widely used for functional genomic applications (see ***[23]*** for review). Usually morpholinos are employed to elicit translational inhibition, although through interruption of splicing events **(Fig. 4)** they also have the capacity to modify splicing reactions, a property that could be useful in defining the role of alternatively spliced mRNAs in megakaryocytes. A drawback is the means of delivery since the preferred option is microinjection; this is obviously not ideal for megakaryocytes, although electroporation might be feasible.

3.1.2. RNA Interference

A method that is stimulating tremendous interest at the moment is that of so-called RNA interference (RNAi). This differs from antisense approaches in that although it involves complementarity between a small RNA molecule and the target mRNA, the active molecule is double-stranded RNA of a defined length (21–22 bp). Specific recognition of the target mRNA elicits its cleavage through an evolutionarily conserved RNase III ("Dicer," ***[24]***). The most effective RNAi molecules are those in which the two strands are linked by a short loop sequence that mimics the structure of an unprocessed intron. Until recently, use of this technique involved injection or uptake of synthetic RNAi molecules into cells. However, the range of possibilities has been increased since it has been shown by several groups (*see*, for example, **refs. *25,26***) that stable expression of an RNAi molecule can be achieved from a plasmid vector using the RNA pol III-dependent promoter from the U6 RNA gene **(Fig. 5)**. This raises

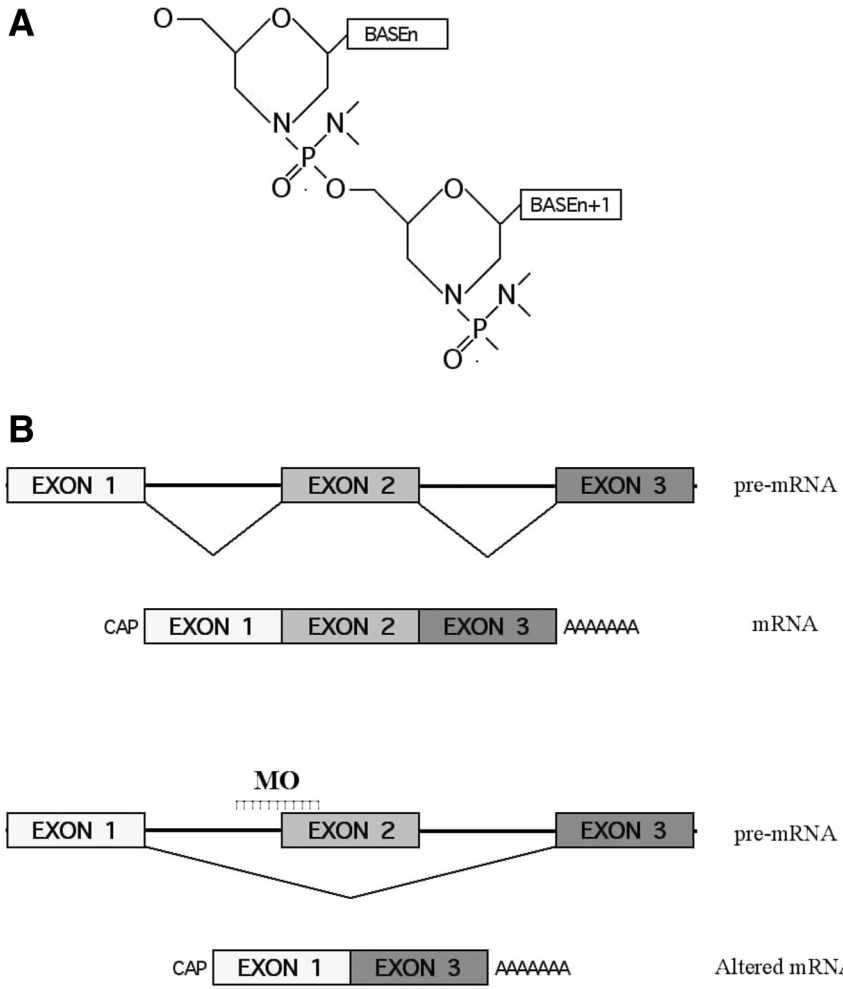

Fig. 4. **(A)** Diagrammatic representation of the basic structural unit of a morpholino oligonucleotide. **(B)** Possible application of morpholino oligonucleotides to investigate alternative spliced mRNAs. The morpholino (MO) is shown annealing specifically at the junction between intron 1 and exon 2 in the pre-mRNA thereby preventing the preferred splicing and leading to a mature mRNA containing only exons 1 and 3.

the possibility that this strategy could be employed in a transgenic context, providing a convenient means to make "knockouts" of genes of interest. Unless a mechanism can be designed to allow imposition of control on such a transgene (perhaps along the lines suggested in **Subheading 2.3.2.**), this approach would have to be limited to genes whose loss of function is not lethal.

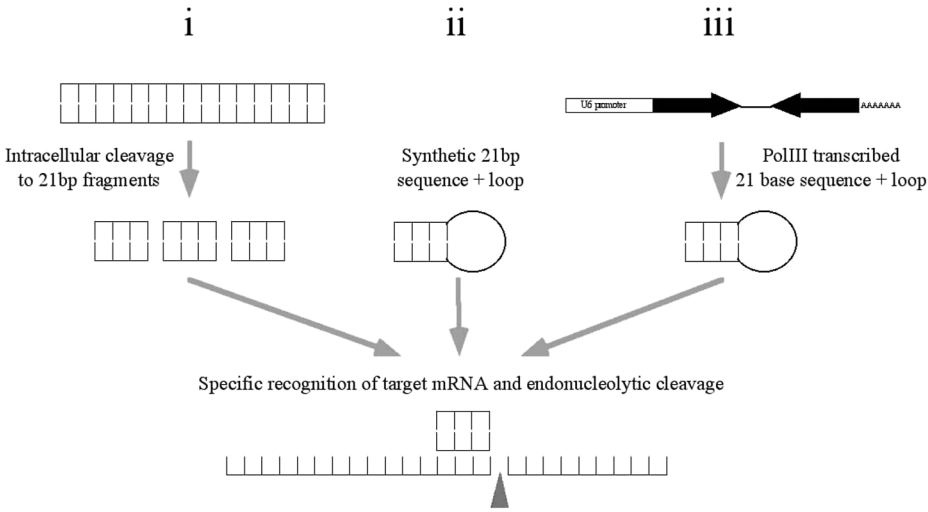

Fig. 5. RNA interference (RNAi). Three routes for the generation of 21-bp double stranded RNAi molecules are illustrated. *(i)* Large double-stranded RNAs are introduced by injection or transfection and are cleaved into fragments by a nonspecific endonuclease. *(ii)* Synthetic oligonucleotides can be introduced by injection or transfection. These are most efficient if the two strands are connected by a small loop. *(iii)* The 21-base sequence and its complement separated by a small "intron" are transcribed from plasmid DNA utilizing a polIII promoter (e.g., from the U6 RNA gene). A short run of Ts at the end of the construct ensures correct termination.

3.2. Removal or Mutation of a Gene

3.2.1. Constitutive Knockout

The most widely adopted approach to removing function of a specific gene involves the irreversible modification, or "knockout," of the chromosomal locus ***(27)***. Ways to achieve a knockout are through the removal of key exons or promoter elements or by insertion of a block that prevents correct transcription, splicing, or translation of the RNA. Careful design of the targeting construct is very important to ensure that gene ablation is complete and that alternative transcription or splicing does not lead to an aberrant product from the modified locus. As described above (**Subheading 2.3.3.**), targeting by homologous recombination involves insertion of vector sequences into ES cells followed by selection for clones carrying the knockout allele **(Fig. 6)**.

Quite a large number of knockouts have been described that have a megakaryocyte/platelet phenotype **(Table 2)**. These knockouts fall into roughly two categories: (1) those that confirmed previously inferred roles for the gene in megakaryocytes and platelets, and (2) those that revealed an unexpected role for the ablated gene product in the megakaryocytic lineage. Examples in the first group are classic megakaryocyte and platelet molecules such as the *c-mpl* gene encoding the receptor for TPO ***(28)*** and the *gpIIb* gene ***(22)***, while

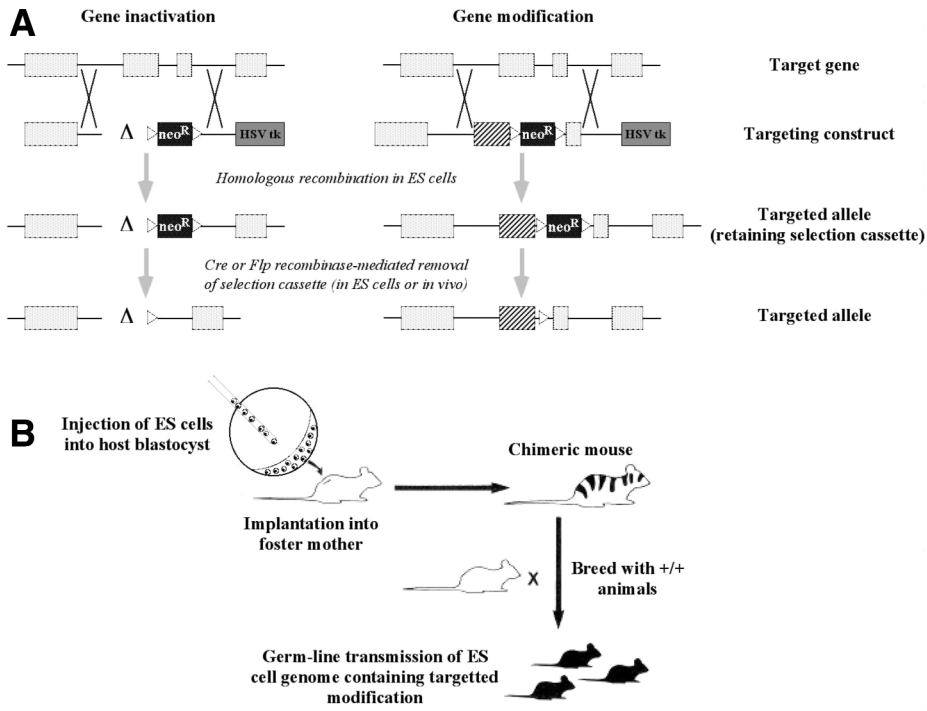

Fig. 6. Gene targeting by homologous recombination in ES cells. **(A)** Two possible strategies are illustrated for modification of a target gene of interest. Coding exons are depicted as light shaded boxes. Homologous recombination between the target gene and the targeting construct is indicated by crosses. On the left, gene inactivation is achieved by removal of sequences including a crucial exon, while on the right, no sequences are deleted but the coding sequence is altered in a particular exon (cross-hatched). Positive and negative selection cassettes (neo^R and HSV-tk) are shown as darker shaded boxes. The open arrows represent recognition sites for either Cre or Flp recombinase. **(B)** Schematic representation of the production of a stable mouse line from a genetically modified ES cell clone. Successful incorporation of the modified ES cells (donor) into the host blastocysts is determined by appearance of the donor coat color (black in this illustration) in the initial chimaeras and following demonstration of germ line transfer.

examples of a gene whose importance in megakaryocyte development was serendipitously revealed is that coding for the NF-E2 transcription factor ***(29)***.

The number of genes successfully knocked out is growing rapidly. The best way to keep up with these is through one of the Web sites that are regularly updated (for example, http://tbase.jax.org or http://research.bmn.com/mkmd).

Table 2
Gene Knockouts With a Megakaryocyte/Platelet Phenotype

Gene	Nature of knockout	MK/platelet phenotype	Reference
FOG-1	Constitutive	Failure of commitment to megakaryopoiesis	***44,46***
Gfi-1b	Constitutive	Failure of megakaryocyte development	***46***
ICAM	Constitutive	Defective megakaryocyte progenitor	***47***
GATA-1	Lineage-specific (enhancer deletion)	Decreased platelets, increased immature megakaryocytes	***40***
Fli-1	Constitutive	Decreased platelets, dysmegakaryopoiesis	***48***
MafG	Constitutive	Decreased platelets, increased megakaryocytes	***49,50***
NF-E2	Constitutive	Decreased platelets	***40***
c-Mpl	Constitutive	Decreased megakaryocytes and platelets	***28,51***
TPO	Constitutive	Decreased platelets	***52***
Bcl-x	Lineage-specific (E/MK-specific Cre-loxP)	Decreased platelets, increased immature megakaryocytes	***37***
VASP	Constitutive	Megakaryocyte hyperplasia and platelet dysfunction	***53***
c-cbl	Constitutive	Megakaryocyte hyperplasia and thrombocytosis	***54***
WASP	Constitutive	Decreased and dysfunctional platelets	***55***
gpIbα	Constitutive	Dysfunctional platelets	***56***
α_{IIb} integrin	Constitutive	Dysfunctional platelets	***22***
β_3 integrin	Constitutive	Dysfunctional platelets	***57***
GPV	Constitutive	Dysfunctional platelets	***58***
PAR 3	Constitutive	Dysfunctional platelets	***59***
PAR 4	Constitutive	Dysfunctional platelets	***60***
PECAM	Constitutive	Dysfunctional platelets	***61***
CD39	Constitutive	Dysfunctional platelets	***62***
LAT	Constitutive	Dysfunctional platelets	***63***
FcγRII	Constitutive	Dysfunctional platelets	***64***
Syk	Constitutive	Dysfunctional platelets	***30,64***
SLP-76	Constitutive	Dysfunctional platelets	***65***
TGF-β1	Constitutive	Dysfunctional platelets	***66***
CGMP kinase I	Constitutive	Dysfunctional platelets	***67***

3.2.2. Conditional Knockout

One of the major drawbacks of conventional knockouts is that if the gene product is essential in many tissues in addition to the cell type of interest, then it is quite likely that the consequence of homozygosity for the mutated allele will be lethality, often at some point during development. Several experimental strategies are employed or have the potential to overcome the problem of lethality.

A possible, although little-explored, solution is to render the mutant allele homozygous in a genetic background in which the gene product is expressed from a transgene only in those cells that are thought to be responsible for the lethal phenotype. As an example, Syk kinase is an important component of signaling in megakaryocytes/platelets; however, the knockout is a perinatal lethal ***(30)*** possibly because defects in vessel endothelial cells lead to serious bleeding problems. If the endothelial defect could be selectively rescued using a *syk* transgene driven by an endothelial-specific promoter (for example, from the *flk-1* gene) then mice might survive adulthood and the influence of the absence of Syk in platelets could be examined.

Another solution, which has been applied in a few cases, is to use hemopoietic cells from the fetal liver of knockout embryos to reconstitute the hemopoietic system of lethally irradiated wild-type animals (so-called radiation chimeras). It is important to ensure that the genetic background of the donor and host are closely matched, if necessary by crossing the knockout strain to the host wild-type strain for at least five generations. Application to the *syk* knockout again provides a good example of the use of this technique ***(31)***.

The solution to the problem of knockout lethality that is rapidly becoming almost standard is to restrict the cell type in which deletion occurs using the Cre-LoxP technology based on bacteriophage recombination systems ***(32)***. Cre recombinase specifically recombines DNA sequences flanked by LoxP sites ("floxed") as illustrated in **Fig. 7A** ***(33)***. The gene of interest is modified in ES cells by homologous recombination as described above. A large number of genes have been modified in this way and many of these will probably be of interest to those studying various aspects of megakaryocyte/platelet biology (*see* **ref.** ***34*** for review of modified genes). Lineage- or stage-specific deletion is then achieved by crossing the floxed gene to a transgenic or knock-in strain that expresses Cre recombinase in a specific cell type **(Fig. 7B)**. Strains expressing a CreER fusion protein can be used for direct activation of Cre activity after feeding animals with estrogen ***(35)***, while a Cre transgene driven from the interferon-responsive Mx promoter can be used to control expression at the transcriptional level by injecting animals with interferon or double-stranded RNA, which induces an interferon response ***(36)***. As more strains become available,

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Fig. 7. *(see opposite page)* Conditional gene ablation using Cre-loxP technology. **(A)** The gene of interest or a critical domain within it is flanked by Cre recombinase recognition sites (loxP), which are indicated by the arrows. When bound by Cre, two sites in the same orientation are recombined resulting in the deletion of the intervening sequences. **(B)** Strategy for the generation of a conditional allele. This is very similar to the scheme

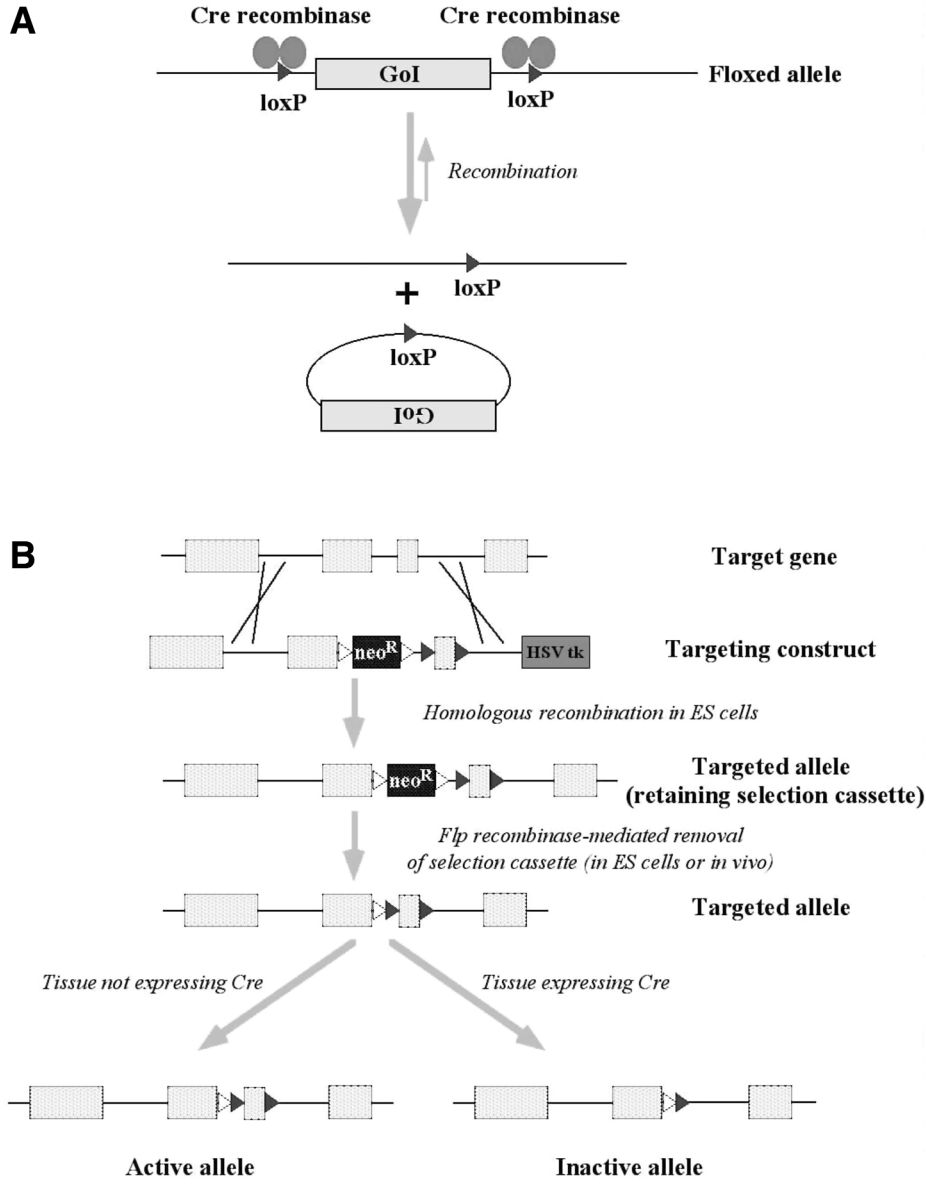

Fig. 7. *(continued)* described in **Fig. 6A**, except that two sets of recombinase recognition sites are introduced. In this example, Flp recognition sites (open arrows) are placed around the neo^R selection cassette to enable its subsequent removal by Flp either in the ES cells or once transmitted to the mouse germ line. Cre recognition sites (shaded arrows) surround the sequences to be conditionally deleted.

lineage-specific expression of Cre recombinase from a transgene or knock-in gene is growing in popularity as a means to control gene deletion. Available strains expressing Cre recombinase can be found in a database compiled by Andreas Nagy (http://www.mshri.on.ca/nagy/Cre.htm).

Cre-LoxP technology shows great promise; nevertheless, there are inherent problems associated with it, the most challenging one remaining the specificity of Cre-recombinase expression. Although knock-ins are conceptually more likely to give the desired pattern of expression, they may be limited in that expression from a single copy might not always generate sufficient Cre protein to elicit efficient recombination. To date, there is no published megakaryocyte-specific Cre-recombinase strain, although a number of laboratories are actively trying to create one. To our knowledge attempts are being made using the *PF4* gene promoter, the *gpVI* gene as a knock-in and, in our own laboratory, the gpIbα gene promoter (J. F., unpublished). In the absence of a perfectly lineage-specific Cre strain, some success has been obtained in deletion of genes in megakaryocytes using mice that express Cre in a limited number of cell types. Hence, floxed alleles of the *bcl-x* and *piga* genes have been specifically recombined in megakaryocytes by making use of the MMTV-Cre ***(37)*** and GATA-Cre ***(38)*** strains, respectively.

As we learn more about the regulatory elements that control lineage-specific expression, it may become possible to achieve lineage-specific gene knockouts by promoter-element deletion rather than deletion of sequences encoding functional domains. An example of such an approach has been described relating to gene ablation in the megakaryocytic lineage. The GATA-1 transcription factor, when deleted constitutively, results in embryonic lethality from anemia due to blocked erythropoiesis ***(39)***. Serendipitously, Shivdasani et al. ***(40)*** found that deletion of a particular upstream regulatory element of the GATA-1 gene caused specific loss of expression in the megakaryocytic lineage and a consequent block to differentiation.

3.2.3. Knockdown

Serendipity has often also led to the identification of gene modifications that produce a reduction in gene expression rather than a complete ablation. In some cases this may have experimental value in determining the function of a gene product. It is difficult at the moment to predict exactly which genetic modifications will produce such a "knockdown" phenotype, although the insertion of the neo^R selection cassette within an intron can often have such an effect. Alternatively, modification of certain promoter elements might produce a reduction in expression levels, as has been seen for the GATA-1 gene knockdown mutation in a promoter proximal regulatory element ***(41)***. Again, prediction of which strategies are likely to be effective in megakaryocytes will become easier as we learn more from this technology and discover more about regulatory mechanisms that control megakaryocyte-specific gene expression.

3.2.4. Knock-In Mutations

Ablation or reduction of gene expression is not the only way in which modification of a gene can be used to explore the function of the encoded product. Homologous recombination in ES cells can also be used to modify specific residues in the coding

sequence so that alternative amino acids are introduced into the protein **(Fig. 6A)**. Production of mutated proteins in this way will be particularly useful as a way of producing animal models for polymorphic variants of proteins thought to be linked to disease susceptibility. More subtle mutations of this sort also provide a potential means for unraveling the function of individual protein domains. For instance, a mouse model was generated to test the role of the cytoplasmic tyrosine motifs in the β_3 chain of the $\alpha_{IIb}\beta_3$ integrin receptor in mediating outside-in signaling by replacing them with phenylalanines ***(31)***.

4. Future Advances

We have tried to summarize many of the most up-to-date advances in technologies that enable manipulation of gene expression in mammalian cells, and which might be particularly useful in relation to work on megakaryocytes and platelets. Most of these techniques are undergoing rapid improvements and we can only wonder at the sophistication that lies around the corner. Certainly, many of the methodologies will become more streamlined and more rapid. Dissemination of the wealth of methods and reagents—for example, available transgenic or floxed gene strains and mice expressing Cre recombinase in specific cells—is bound to facilitate advances. Combination of manipulated gene expression in megakaryocytes with analysis of the transcriptosome or proteome is also likely to become common practice. Our discussion of methodologies has centered on application to already partially characterized genes, but of course there are bound to be many more genes relevant to megakaryocyte/platelet biology that are yet to be identified. Candidates will emerge from microarray screening of expressed sequences and proteomic analyses, but there must also be a huge potential for screening for mouse mutations, probably recessive, that influence various aspects of megakaryopoiesis and platelet function. Many such large-scale projects are either planned or under way ***(42,43)*** and it is hoped that the phenotypic screening strategies that are employed will also include examination for effects at the level of platelet numbers and function.

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