The expression patterns of genes involved in the RNAi pathways are tissue-dependent and differ in the germ and somatic cells of mouse testis

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Summary

Different RNA interference (RNAi) components participate in post-transcriptional regulation via RNA silencing. The expression pattern of the genes *Drosha*, *Dicer* and the members of the Argonaute family *Ago1*, *Ago2*, *Ago3* and *Ago4*, all elements participating in the RNAi pathways, were investigated in mouse somatic tissues and testis using quantitative RT-PCR. Expression patterns of different testis cells and those emerging during testis development were also investigated. The differential patterns of expression seen suggest potential pleiotropic roles for certain components of the RNAi machinery. Both spermatocytes and spermatids showed a defined gene expression pattern. The strong expression of Ago4 in germ cells suggests this protein plays a key role in germ cell differentiation in the seminiferous epithelium.

Introduction

RNA interference (RNAi) is mediated by small interfering RNAs (siRNAs) and microRNAs (miRNAs) [1]. In differentiation and development, RNAi operates as a regulator of gene expression [2]. Such regulation is probably more important in complex processes - certainly the gene expression that governs the events in male germ cell differentiation requires very precise control [3] [4]. RNAi mechanisms may act throughout spermatogenesis [5] [6]; in support of this, Yu et al. (2005) [7] report several miRNAs to be differentially expressed during the development of the seminiferous epithelium.

Although the intricate process of gene regulation by RNAi is not fully understood, many conserved elements participating in the RNAi pathway have been identified [8]. This pathway involves two main steps: (1) the processing of endogenous miRNA or dsRNA by the RNaseIII enzymes Drosha and Dicer, and (2) the cleavage of target RNA via the RNA-induced silencing complex (RISC) (see the recent review by Hammond 2005)[9]. Argonaute proteins - components of RISC - are highly basic proteins [10] characterized by two domains: the PAZ domain (which interacts with the 3' end of siRNAs) and the PIWI domain (which binds to the 5' end of siRNAs as well as the target RNA to be silenced). The Argonaute family can be subdivided into the Ago and Piwi subfamilies. The Ago members are generally considered to be ubiquitously expressed and to be involved in siRNA and miRNA function [11]. The expression of the Piwi members is more restricted to germ line cells [12]. This is based on the isolation, from mouse testis, of a new class of small RNAs known as piwi-interacting RNAs (piRNAs) [13-16]. AGO1 and AGO2 form complexes with Dicer. AGO2 alone, however, is responsible for the cleavage activity of RISC, presumably through the RNAse H like structure in the PIWI domain [17]. AGO1 AGO2, AGO3 and AGO4 have been identified in mRNA decay centers, or processing bodies (p-bodies) as they are known in somatic cells. It has been suggested that AGO proteins act through mRNA degradation or via

the inhibition of translation [18]. Recently, Kotaja et al. (2006) [19] localized Dicer, AGO2, AGO3 and Miwi (a murine Piwi family member) along with miRNAs in germ-cell specific cytoplasmic chromatoid bodies (c-bodies). These structures are similar to p-bodies and are thought to be involved in the storage and processing of haploid cell transcripts.

In mammalian testis, different types of small RNAs may operate as modulators of gene expression. However, the participation of RNAi machinery elements in specific cell differentiation programs such as spermatogenesis has not been established. To investigate the participation of RNAi machinery elements in spermatogenesis, the expression of the genes that encode Drosha, Dicer, AGO1, AGO2, AGO3 and AGO4 (officially known as *Rnasen, Dicer1, Eif2c1, Eif2c2, Eif2c3* and *Eif2c4*) was analyzed (using quantitative RT-PCR [qRT-PCR]) in the developing testis, in different seminiferous epithelium types, in a number of somatic tissues, and in different seminiferous epithelium cells types.

Methods and Materials

Tissue collection and cell isolation

Animals were treated according to the guidelines of the CSIC Bioethics Committee. A CD-1 mouse colony was maintained in a temperature- and humidity-controlled room. Food and water were provided *ad libitum*. Somatic tissues (brain, spleen, heart, muscle and lung) were obtained from adult mice. Whole testes were obtained from mice at days 6, 10, 14, 18 and 22 postpartum (dpp), and from adults.

Primary Sertoli cells were cultured from the testes of mice aged 16-18 dpp. All cultures were maintained for 2 weeks at 37ºC in a 5% CO_2/95% air atmosphere and in Dulbecco's modified Eagle's medium:Ham-F12 medium (Gibco, BRL) (1:1), following standard procedures [20]. Pachytene spermatocytes, round spermatids and elongating

spermatids from adult mice were enriched using BSA density gradients on STA-PUT [21]. The purity of all isolated and cultured cells was 90–95%, as determined by morphological criteria and RT-PCR using cell-type specific primers as previously described [22]. Mouse NIH-3T3 fibroblasts were cultured in a 5% CO_2/95% air atmosphere at 37ºC in DMEM supplemented with 10% heat inactivated fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 10 μg/ml streptomycin.

Total RNA extraction and analysis of mRNAs by qRT-PCR

Total RNA from testicular and somatic tissues and from isolated testicular cell types was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Gene expression levels were determined by real time RT-PCR using the ABI Prism 7700 Sequence Detection System (Applied Biosystems). Residual genomic DNA was removed from total RNA by treatment with RQ1 RNase-free DNase (Promega). RNA was reverse transcribed using a 17-mer oligo-dT primer and SuperScript II Reverse Transcriptase (Life Technologies). PCR was performed in a final volume of 25 μl using SYBR Green PCR Master Mix (Applied Biosystem) and 100 nM of each primer. Primer combinations for the specific amplification of the analyzed genes were designed using Primer Express software (version 1.5, Applied Biosystem), following the method of Livak (1999) [23]. Table 1 lists the primers used in PCR.

The PCR reaction conditions were as follows: 40 cycles of denaturation at 95ºC for 15 s, annealing and extension at 60ºC for 1 min, and a final extension step at 72ºC for 1 min. The PCR products were also examined by melting curve analysis and agarose gel electrophoresis. A replicate was run for each sample omitting the reverse transcription step as a template-negative control. Each experiment was repeated at least three times. The amount of ribosomal protein S16 (*Rps16*) mRNA [24] was measured in each sample for normalization purposes.

Gene expression data during seminiferous epithelium development were presented using a modification of the $2^{-\Delta\Delta Ct}$ method [25] [26]. Expression in adult testis was used as the calibrator. The data for tissues and cell types normalized only to *S16*, without the use of a calibrator are presented.

Statistical analysis

Since the range of gene expression values calculated with the $2^{-\Delta\Delta Ct}$ method is asymmetrically distributed [26], the values recorded were log-transformed to generate a normal distribution. A student t test was then used to compare the mean expression of each gene for each somatic tissue with mean expressions for the testis ($p<0.05$). Pearson Correlation coefficients (r) were calculated to determine the relationship between the expressions of paired genes in the different tissues analyzed. The value of the determination coefficient (r^2) for each pair of genes was determined to examine the degree of dependence between them.

Results

Differential expression of RNAi components in testis and somatic tissues

The expression of *Drosha*, *Dicer* and *Ago1*, *2*, *3* and *4* was different in the different tissues analyzed. The highest levels of expression were generally seen in the testis and brain (Fig. 1). *Ago4* was most strongly expressed in the testis, while the expressions of *Drosha*, *Dicer*, *Ago2* and *Ago3* were significantly stronger in the brain than in the other tissues. *Ago1* was the gene most strongly expressed in the spleen. The expression pattern of these RNAi component genes is therefore tissue-specific.

To determine whether these genes are co-regulated, the Pearson correlation coefficients (r) and determination coefficients (r^2) for paired genes were calculated (Table 2). A positive, significant correlation was observed between *Drosha-Dicer* (R^2=0.69, $p<0.05$), *Drosha-Ago2* (R^2=0.69, $p<0.05$), *Drosha-Ago3* (R^2=0.72, $p<0.05$) and *Dicer-Ago3* (R^2=0.77, $p<0.05$). No significant correlation was seen between *Ago1* or *Ago4* and other genes. However, when somatic tissues only were taken into account, significant correlations between *Ago4* and *Ago2* (R^2=0.87, $p<0.05$), and between *Ago4* and *Ago3* (R^2=0.96, $p<0.05$) were observed.

Analysis of gene expression during mouse spermatogenesis

Given the continuous cell differentiation process associated with spermatogenesis, the regulation of RNAi-related genes in seminiferous epithelium cells and during testis development was analyzed. Enriched populations of pachytene spermatocytes, round and elongating spermatids, Sertoli cells and non-testicular somatic NIH-3T3 fibroblasts were all analyzed. The qRT-PCR results revealed a different pattern of expression for germ cells and somatic cells (Fig. 2). While *Drosha* was the most abundant transcript in germ cells, *Ago2* was the most common in both Sertoli and NIH-3T3 cells. The other genes analyzed showed similar levels of expression in Sertoli and NIH-3T3 cells. In contrast to the somatic cells, spermatocytes and spermatids showed a similar pattern of expression for the genes analyzed. A relatively high level of *Ago3* and *Ago4* was detected in pachytene spermatocytes, where it is assumed that the maximum level of gene regulation in spermatogenic cells occurs.

Expression during several testis developmental stages - at 6, 10, 14, 18, 22 dpp - and at adulthood was analyzed (Fig. 3) to capture the expression of RNAi-related genes during the first wave of spermatogenesis [27]. The expression of *Drosha* was increased from 14 to 18 dpp in agreement with the higher levels seen in germ cells than in Sertoli cells (Fig. 2).

However, most of the expression levels of the different RNAi-related genes analyzed showed modest variations that correlated with the established proportions of the different cell types appearing during testis development [27]; the relative proportion of germ cells increases in the pubertal development of the testes. The clearest increase in gene expression during development was detected for *Ago4,* which was only weakly detected in the Sertoli and somatic NIH-3T3 cells.

Discussion

The paucity of antibodies against mammalian proteins involved in RNAi machinery is currently limiting a parallel assessment of protein accumulation to corroborate the differential expression patterns observed in tissues and development. Recent monoclonal antibodies raised against Argonaute proteins recognized all Ago proteins without discrimination between the four of them [28, 29].

The Drosha and Dicer proteins have been studied extensively, but the functions and interactions of Argonaute proteins are not fully understood (for a recent review see [11]). The expression of the most studied elements in the RNAi machinery shows high variability not only between different tissues, as reported by Sago et al. [30], but also between different cell types of the same tissue such as those of the seminiferous epithelium. The pattern of gene expression described by Sago et al. [30] for *Drosha*, *Dicer, Ago1*, *Ago2*, *Ago3* and *Ago4* differs to that seen in the tissues studied here. This might be due to the use, by Sago et al. [30], of the *G3pdh* gene as the normalization control, or the use of a non-defined developmental stage for the tissue analyzed. It has been shown that the usefulness of a housekeeping gene as an internal control in qRT-PCR depends on the tissue and/or developmental stage. However, due to the high variability of *G3pdh* expression, this gene does not provide an appropriate control [31] [32]. Ribosomal protein S16 [33] was therefore

used in the present study. However, *Ago3* and *Ago4* were shown in both studies to be highly expressed in the testis compared to other tissues. The high expression levels of *Drosha*, *Dicer*, *Ago2* and *Ago3* observed in the brain with respect to other tissues may be related to miRNA pathway activation during brain development [34] [35] [36] [37] as well as the regulation of mRNA translation in developing axons [38] and dendrites [39].

The Pearson correlation analysis of paired genes suggests co-regulation of some elements of the RNAi pathway (Table 2). Taking into account the data for all tissues analyzed, *Drosha* expression showed a significant, positive correlation with that of *Dicer*, *Ago2* and *Ago3*, but no correlation was detected with *Ago1* or *Ago4*. The divergent expression of *Ago1* in spleen and *Ago4* in testis might explain this since, when only somatic tissues are taken into account, *Drosha* and *Ago4* expressions are correlated. Similar interpretations might be made for *Dicer* and the genes encoding the Ago proteins. The lack of complete correlation between paired genes agrees with data on the involvement of RNAi elements in cell cycle, apoptosis, differentiation and development pathways. Evolutionary functional diversification from ancestral RNAi machinery in new RNAi-independent pathways has been recently postulated [40].

The different expression patterns observed in germ and somatic Sertoli cells are quite remarkable. The high expression of *Drosha* and *Ago4* contrasts sharply with that of *Ago2* in the Sertoli cells. Further, Sertoli cells and NIH-3T3 fibroblasts showed a similar pattern (except for the higher expression of *Dicer* in Sertoli cells). Drosha is an essential protein in the biogenesis of endogenous miRNAs that are processed by Dicer and incorporated into the RISC pathway, while Dicer appears to be a common denominator in both siRNA and miRNA pathways. The larger amount of *Drosha* transcripts in germ cells than in Sertoli cells supports the hypothesis that germ-line differentiation processes may require the participation of miRNAs, some of which have recently been identified as testis-specific [7]. This hypothesis

agrees with the observation that tissues that undergo terminal differentiation coordinated by changes in gene expression may be in part tightly regulated by miRNAs; this has been suggested with respect to cell determination during embryonic development [41].

In *Arabidopsis*, Ago4 has been associated with RNA-directed DNA and histone methylation mediated by a class of siRNAs [42-46]. In mammals, changes in the methylation pattern have been identified as important epigenetic control mechanisms during spermatogenesis [47] [48]. However, the homology between *Arabidopsis* and mouse *Ago 4* is low, except for the Argonaute domains. It has been postulated that RNAi components in mammalian cells may be used exclusively for miRNAs pathways, and, unlike in plants, DNA methylation may require input from no RNA component under normal conditions [49]. If an adaptive response to RNAi only occurs in germ cells (due to the absence of an interferon response to dsRNA), then in mammals some Ago protein might participate in a pathway similar to that seen in *Arabidopsis*. In fact, members of the Argonaute family in mouse - Miwi [14], Mili [13] - have recently been reported essential in meiotic progression through their binding a new class of small RNAs called piRNAs (piwi-interacting miRNA). Miwi2 (a Piwi –family member) has been clearly associated to the specific control of transposons during differentiation of male germ cell in mouse [50], suggesting a conserved function for Piwi proteins [51]. Nevertheless, the precise functions of piRNAs and associated proteins are yet to be defined. The present results suggest a potential role for Ago4 in male germ cells in mammals.

The differential pattern of gene expression seen in this work for the Drosha, Dicer and Argonaute proteins in the different tissues and cell types suggests differential participation by the elements of the RNAi machinery in the complex and probably cell/tissue-specific regulatory mechanisms of post-transcriptional gene regulation.

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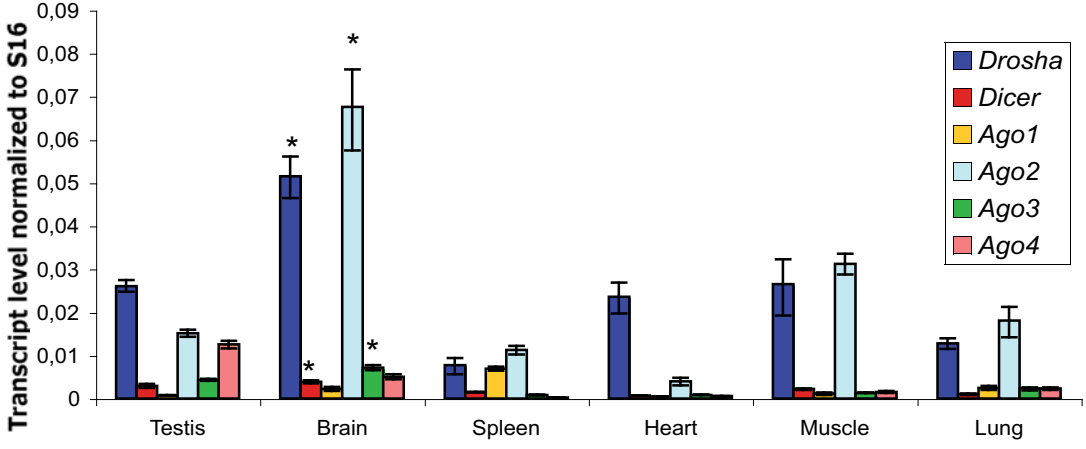
Figure legends

Fig. 1. Quantitative RT-PCR analyses of *Drosha*, *Dicer*, *Ago1*, *Ago2*, *Ago3* and *Ago4* transcripts in different tissues. Error bars represent standard deviations. Asterisks indicate statistical significance.

Fig. 2. Quantitative RT-PCR analyses of *Drosha*, *Dicer* and *Ago1-4* transcripts in cell types from the seminiferous epithelium (pachytene spermatocytes, round spermatids, elongating spermatids and Sertoli cells) and in the somatic cell line NIH-3T3. Error bars represent standard deviations.

Fig. 3. Quantitative RT-PCR analyses of *Drosha*, *Dicer*, *Ago1*, *Ago2*, *Ago3* and *Ago4* transcripts during postnatal testis development (dpp: days post partum; Ad: adults). Error bars represent standard deviations.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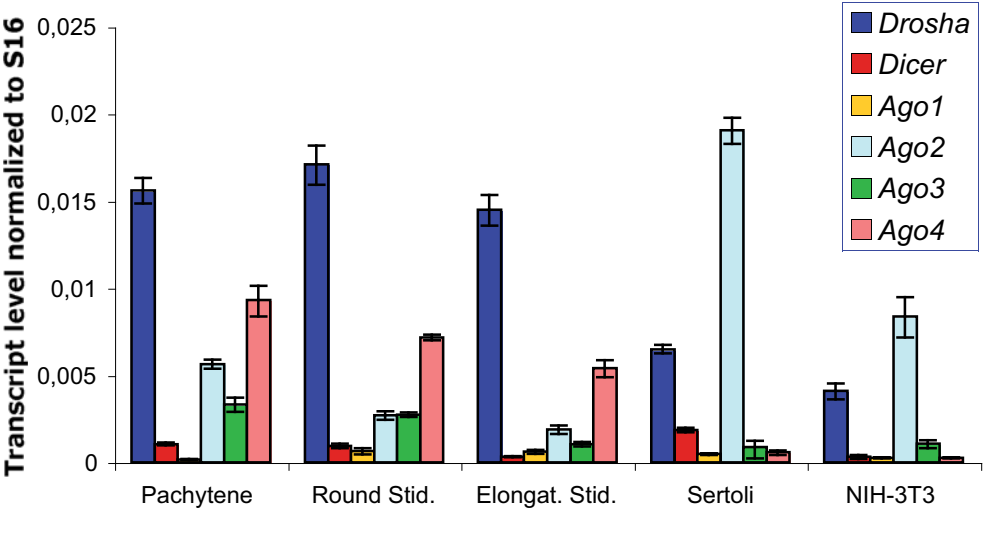
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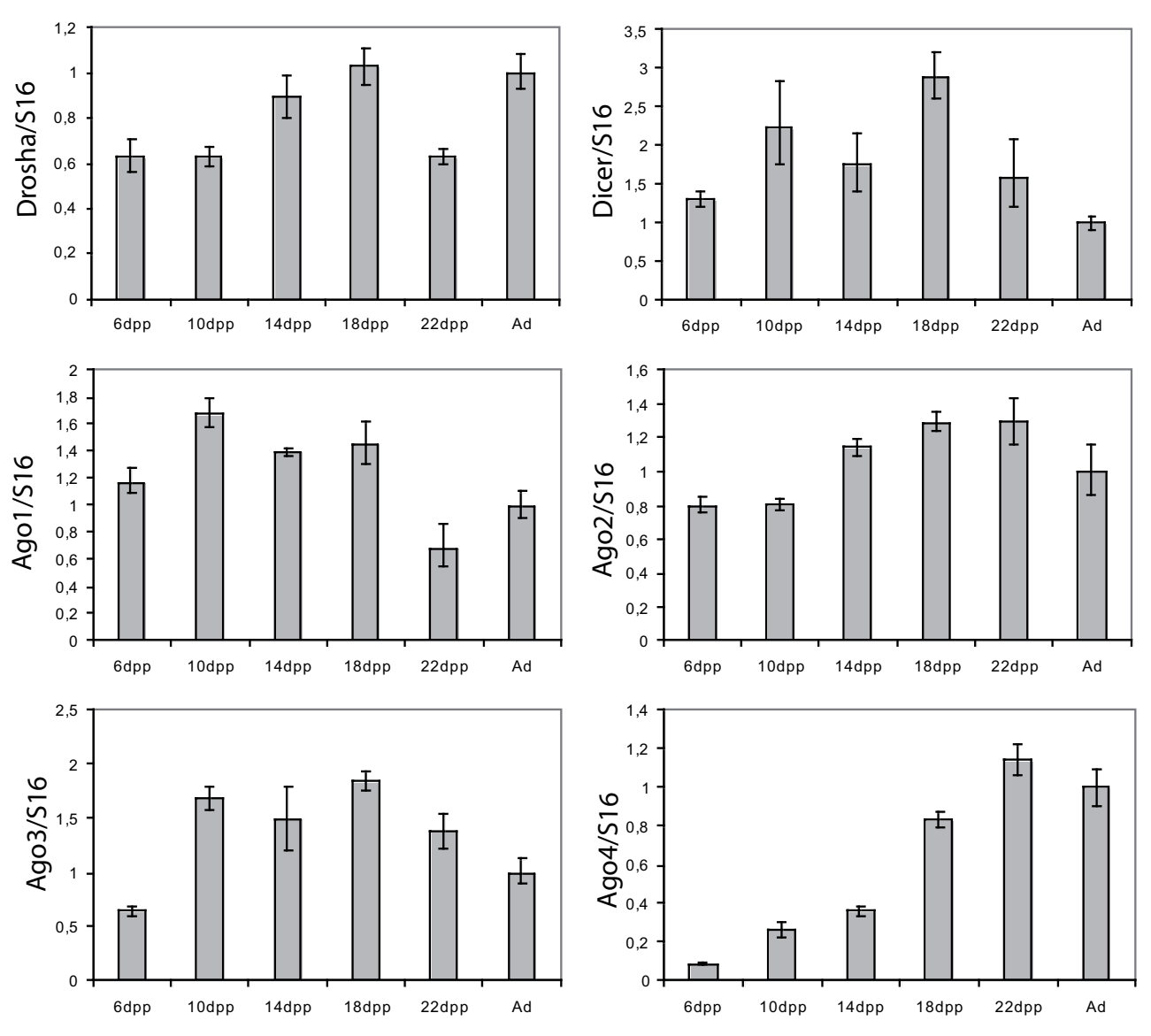
Drosha/S16
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14dpp
18dpp
22dpp
Ad
Dicer/S16
3,5
3
2,5
2
1,5
1
0,5
0
6dpp
10dpp
14dpp
18dpp
22dpp
Ad
Ago1/S16
2
1,8
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
6dpp
10dpp
14dpp
18dpp
22dpp
Ad
Ago2/S16
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
6dpp
10dpp
14dpp
18dpp
22dpp
Ad
Ago3/S16
2,5
2
1,5
1
0,5
0
6dpp
10dpp
14dpp
18dpp
22dpp
Ad
Ago4/S16
1,4
1,2
1
0,8
0,6
0,4
0,2
0
6dpp
10dpp
14dpp
18dpp
22dpp
Ad